Elicitation of Casbene Synthetase Activity in Castor Bean

THE ROLE OFPECTIC FRAGMENTS OF THE PLANT CELL WALL IN ELICITATION BY A FUNGAL ENDOPOLYGALACTURONASE

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Endopolygalacturonase isolated from culture filtrates of the fungus *Rhizopus stolonifer* was shown previously to act as an elicitor of biosynthetic capacity for the antifungal agent, casbene, in castor bean (*Ricinus communis* L.) seedlings (S.-C. Lee, C.A. West 1981 Plant Physiology 67:633-639). Selective amidation of exposed carboxyl groups of the pure fungal endopolygalacturonase using intermediate activation with a water-soluble carbodiimide under mild conditions leads to inactivation of its enzymatic activity. Tests of active and partially inactivated preparations of the enzyme reveal a close correlation between the levels of catalytic and elicitor activities. This suggests that the catalytic activity of the enzyme is necessary for its function as an elicitor. Treatment of the cell-free particulate fraction of homogenates of castor bean seedlings with the active fungal endopolygalacturonase results in the production of a heat-stable, water-soluble component which is highly active as an elicitor of casbene synthetase activity. Several additional lines of evidence, including the susceptibility of the heat-stable elicitor fraction to partial inactivation following prolonged treatment with endopolygalacturonase, indicate that the heat-stable elicitor is most likely a pectic fragment of the plant cell wall and that it is a required intermediate in the process of elicitation of casbene synthetase activity by the fungal endopolygalacturonase.

ABSTRACT

Many species of higher plants have been shown to produce antifungal compounds known as phytoalexins (4, 6, 10). The presence of low or undetectable levels of phytoalexins in healthy plant tissues and the greatly increased accumulation of these substances in the region of fungal infection are distinctive features of phytoalexin production. Because of these characteristics and their general toxicities for a broad range of fungi, phytoalexins are believed to play a defensive role against invasion of the plant by fungal predators.

Casbene is one of the five cyclic diterpene hydrocarbons produced from GGPP in cell-free extracts of young seedlings of the castor bean (*Ricinus communis* L.) (14, 15). Extracts of healthy seedlings produce little or no casbene, whereas extracts of seedlings exposed for several hours to any of several phytopathogenic fungi show a greatly enhanced capacity for casbene synthesis (18). This characteristic coupled with demonstration of the antifungal properties of casbene led to the proposal that casbene may serve the castor bean plant as a phytoalexin (18). A number of the biochemical characteristics of casbene biosynthesis have been elucidated (26).

Substances which trigger the production of phytoalexins in higher plants are termed elicitors. A wide variety of fungal molecules, including polysaccharide wall components, polypeptides, glycoproteins and lipids, have been implicated as elicitors (25). Two lines of investigation have shown that fungal enzymes can also act as elicitors. Swinburne (22, 23) has implicated a fungal proteinase as an elicitor of benzoic acid production in infected apple fruit. In addition, recent work in our laboratory (11, 12) has shown that homogeneous α-1,4-endopolygalacturonase from culture filtrates of the fungus *Rhizopus stolonifer* elicits casbene synthetase activity in castor bean (*R. communis* L.) seedlings. In both cases, heat-treatment of the enzyme preparations leads to equivalent losses of both enzymic activity and elicitor ability. This suggests that the elicitor abilities of these enzymes may be dependent on their catalytic activities.

The work described in this paper demonstrates that pectic cell wall fragments released from particulate fractions of castor bean seedling homogenates through the action of *R. stolonifer* endopolygalacturonase possess casbene synthetase elicitor activity and supports a model in which these elicitor fragments serve as obligate intermediates in the process of elicitation by the enzyme *in vivo*.

MATERIALS AND METHODS

**Elicitor Bioassay for Casbene Synthetase.** The elicitor activity of various test solutions was measured using a bioassay for the levels of casbene synthetase activity in treated seedlings as described by Lee and West (11). Castor bean seeds were shelled, surface-sterilized in a dilute solution of NaOCl, and germinated under sterile conditions at room temperature for 53 to 55 h. For each test solution, 10 seedlings with the radicles removed were cut along the plane dividing the cotyledons with a double-edged razor blade and placed, cut surface down, in a sterile Petri dish containing two layers of Whatman GF/A glass fiber filter and a single layer of cheesecloth. The test solution (10 ml) was added to the Petri dish which was covered and incubated for 10 to 12 h at 25°C in the dark.

After incubation, the set of pooled seedlings from one treatment was homogenized in cold homogenization buffer. The homogenates were centrifuged and portions of the supernatant were removed and assayed for casbene synthetase activity. In a typical assay, 100 µl enzyme extract and 300 µl buffer were incubated with 100 µl 50 µM radiolabeled GGPP at 30°C for 30 min. The reaction was quenched with ethanol and the casbene was extracted with petroleum ether. The casbene was separated from other substances by TLC and quantitated by measuring the radioactivity associated with the appropriate region of each plate.

Some casbene synthetase activity was observed in seedlings which had been subjected to the experimental assay procedure,
but which had not been treated with elicitor. These control values generally ranged between 5,000 and 6,000 dpm in casbene when 4,4′-[3H]GGPP was used as the substrate in a 30-min assay. A control was run with each set of test samples and the control value was subtracted from the test sample values. Under optimal conditions, elicitor-treated seedlings could accumulate up to five times the control level of casbene synthetase activity. A typical dose-response curve with Rs EPGase as the elicitor is shown in Figure 1. At the lower concentrations of elicitor, the amount of casbene synthetase activity follows a nearly linear relationship with the amount of elicitor tested. Higher levels of elicitor, however, saturate the response. The amount of EPGase tested in elicitor bioassays was usually between 0.2 and 0.3 units in order to produce responses which were in the approximately linear region of the dose response curve.

Synthesis of Radiolabeled Geranylgeranyl Pyrophosphate. 4,4′-[3H]Geranylgeraniol (10.0 mCi/mmol) was synthesized from farnesylacetone in a manner similar to that described by Upper and West (24) as later modified by Simcox (17). The tritium label was introduced into farnesylacetone by refluxing a solution of farnesylacetone and tritiated water in dioxane for 40 h. A small amount of NaOH was added to the mixture prior to reflux to catalyze the exchange of hydrogens bound to carbons alpha to the carbonyl function with tritium atoms of the tritiated H2O. A water-cooled condenser was kept in place above the reaction vessel and the reaction mixture was maintained under dry nitrogen throughout the period of refluxing. Pyrophosphorylation of the alcohol was achieved with the method recently introduced by Dixit et al. (5) in which tetra (tetraethylammonium) pyrophosphate is employed as a pyrophosphorylating agent with geranylgeranyl bromide. The tritiated GGPP synthesized in this manner was used in all reported experiments except where specifically indicated.

Purification of Endopolygalacturonase from Rhizopus stolonifer. EPGase from culture filtrates of R. stolonifer was purified to homogeneity following the techniques described by Lee and West (11). The purification sequence involved chromatography of the concentrated culture filtrate on Sephadex G-25, G-75, and CM-50 under conditions suitable for the preservation of enzyme activity. All samples of Rs EPGase employed in this work were purified to homogeneity in this manner. In some instances, the homogenate enzyme was chromatographed on Sephadex G-10 (coarse grade) in 25 mm NaCl when EPGase in a low salt solution was required.

EPGase activity was quantitated by measuring the rate of liberation of reducing termini from 0.25% solutions of polygalacturonic acid at pH 4.9. Reducing ends were measured using the Nelson-Somogyi spectrophotometric assay (13, 19). A unit of enzyme was defined as that amount which would catalyze the hydrolysis of 1 μmol galacturonosidic bonds/min from 0.25% polygalacturonic acid at pH 4.9 and 30°C.

Preparation and Fractionation of Castor Bean Homogenate. Frozen castor bean seedlings which had been germinated for 55 h were homogenized in three times their weight of cold 10 mm histidine buffer (pH 6.3) in a Waring Blendor or VirTis “23” homogenizer at top speed for 2 min. This homogenate was then centrifuged at 37,000g for 15 min. The supernatant was carefully decanted and recentrifuged at 37,000g for another 15 min. The supernatant from this second spin was employed as the S15 fraction of the homogenate.

The pellet obtained from the first centrifugation was resuspended in a small amount of the same homogenization buffer by brief homogenization and then subjected to recentrifugation at 12,000g for 10 min. The pellet from this centrifugation was defined as the P12 fraction of the homogenate and was typically used as a 10% (w/v) suspension in the same buffer.

Preparation of Heat-Stable Elicitor from Castor Bean Tissue. The P12 fraction of the castor bean seedling homogenate was routinely used as the source of heat stable elicitor. To a 10% (w/v) suspension of castor bean P12 fraction in 10 mm histidine buffer (pH 6.3) was added a sufficient amount of a solution of Rs EPGase (usually a 1% v/v aliquot of a stock solution containing 100 units/ml of Rs EPGase) to yield a final enzyme concentration of 1 unit/ml in the incubation sample. This suspension was then incubated for 4 h on a rotary shaker at 30°C. At the end of this period, the suspension was centrifuged at 37,000g for 15 min. The supernatant containing the heat-stable elicitor was carefully removed and heat-treated at 100°C for 20 min to inactivate the EPGase. This solution was either used directly as a source of heat-stable elicitor or concentrated by lyophilization before use.

Heat Inactivation of Rs EPGase. In many circumstances, the destruction of excess EPGase activity in particular solutions was necessary. This was accomplished by heat-treating the solutions at 100°C for 20 min in a boiling water bath. Tests showed that no detectable EPGase or EPGase-associated elicitor activity remained in solutions treated in this manner.

Chemicals and Biological Materials. Citrus pectin and polygalacturonic acid (Grade III) were obtained from Sigma. Glycine, ethyl ester, EDC, and tomato pectinesterase were also obtained from Sigma. Farnesylacetone was a generous gift from Hoffman-La Roche Pharmaceutical Co., Basel, Switzerland.

Cultures of Rhizopus stolonifer were grown in 2.8 L Fernbach flasks in a liquid glucose/asparagine/salts medium for 4 days at 30°C as described by Lee and West (11). Culture filtrates were prepared by passing the spent medium through Whatman No. 1 filter paper and then, successively, through Millipore 1.2, 0.45 and 0.22 μ filter membranes.

Castor bean seeds were obtained from castor bean plants grown in the UCLA Botanical Gardens and were from either the October 1978 or September 1980 harvest.

RESULTS

The Requirement of Enzymic Activity for Rs EPGase Elicitor Activity. Previous evidence from our laboratory has shown that treatments which destroy the enzymic activity of solutions of Rs EPGase such as heat, pronase digestion, or periodate oxidation similarly destroy the elicitor activity of these solutions (12, 21). One possible explanation for these observations is that the enzymic activity of Rs EPGase is required for its elicitor ability. To test this hypothesis, it would be useful to have a preparation of Rs EPGase for which the enzymic activity had been destroyed in
some manner with only minimal disruption of its tertiary structure, water solubility, and external carbohydrate structure. An inactivation procedure which should meet these criteria employs covalent modification of the exposed carboxylic acid groups of active Rs EPGase using a water-soluble carbodiimide as an intermediate activating agent.

Covalent modification of carboxyl groups was achieved by the method of Hoare and Koshland (9). Homogeneous Rs EPGase in 25 mM NaCl was prepared as described under “Materials and Methods.” To 5 ml of a solution containing 500 units of EPGase and 0.5 mM glycine ethyl ester was added solid EDC to a concentration of 0.1 M. The pH was held constant at 4.75 in a Radiometer pH-stat by titration with 0.1 N HCl at room temperature. Under these conditions, exposed carboxyl groups on the native enzyme should react with the carbodiimide to form intermediate O-acylisourea derivatives. These intermediates then undergo nucleophilic attack by the amino group of glycine ethyl ester resulting in the conversion of free carboxyl groups into stable amides. After 1 h of incubation, the modification was stopped and the excess EDC was destroyed by the addition of 1.0 M sodium acetate (pH 4.75) in 2-fold M excess to the initially added EDC. An assay for enzyme activity at this point showed that approximately 40% of the original enzyme activity had been lost.

The reaction mixture was loaded on a 2.5 × 40 cm column of Sephadex G-10 (coarse grade) equilibrated in 25 mM NaCl and then eluted with 25 mM NaCl. The void volume fractions (marked by a sharp A280 peak) contained the soluble protein which was effectively separated from included fractions containing the unwanted reagents. The three fractions (10 ml) containing the most protein were pooled and brought to 0.5 M in hydroxyamine by the addition of 3.0 M hydroxylamine at pH 7.0 and allowed to stand at room temperature for 5 h. This treatment has been shown to regenerate tyrosine residues that may have been activated by the carbodiimide reagent during the covalent modification procedure (3).

A method was employed for enriching the solution in inactivated enzyme which made use of the differential affinities of active and inactive enzyme for PGA. Ten ml of the covalently modified enzyme solution, which had been raised to 20 mM citrate/100 mM NaCl and adjusted to pH 3.1 with cold 6 N HCl, was applied to a 1 × 2 cm column of PGA which had been packed and washed in 20 mM citrate/100 mM NaCl (pH 3.1). The column was eluted with the same buffer solution. The fractions containing the majority of the material absorbing at 280 nm were pooled and applied to a 2.5 × 40 cm column of Sephadex G-50 (coarse grade) equilibrated in 25 mM NaCl. A symmetrical protein peak coinciding with the void volume eluted first and was well separated from the fractions containing the unwanted included molecules whose position was indicated by conductivity measurements. A pool of five void volume fractions (17 ml) containing the highest concentrations of protein constituted the final solution of inactivated Rs EPGase.

Solutions of the resulting mixture of inactivated and completely active EPGase were tested for enzyme activity. Estimates of the protein concentration of each solution were made from the A_{280} measurements and an approximate extinction coefficient at 280 nm calculated from amino acid analysis of the pure protein (12). The results indicated that the covalent modification and enrichment procedures resulted in a solution of EPGase whose specific enzyme activity was 32% of that of the initial, fully active enzyme preparation. The enrichment procedure using the PGA column resulted in the removal of about two-thirds of the active enzyme and a final preparation which was 1.7 times enriched with respect to inactivated protein.

Quantitative elicitor bioassays of active and partially inactivated enzyme preparations were performed to test whether the elicitor ability of Rs EPGase was a function of its enzymic activity or of its externally recognizable molecular structure (Table I). A sample containing inactivated enzyme which had been heat-treated before use was also tested to monitor for the presence of any residual pectic elicitor which might have been released from the PGA column used in the enrichment step and could therefore interfere with the results. No significant elicitor activity was associated with the heat-treated control sample, thus indicating that little pectic material with elicitor activity which might have eluted from the PGA column remained with the fraction of inactivated enzyme after Sephadex G-50 chromatography.

A visual representation of the elicitor bioassay data is provided as a histogram in Figure 2. From this method of presenting the results, it can be readily seen that the elicitor activity of the various Rs EPGase preparations appears to be much more closely correlated with the amount of enzymic activity they possess than with the total amount of protein present.

Production of a Water-Soluble, Heat-Stable Elicitor by the Action of Rs EPGase. The preceding experiment demonstrates the importance of the enzymic activity of Rs EPGase for its function as an elicitor. This suggests the possibility that digestion fragments resulting from the hydrolytic action of EPGase may play a role in the process of elicitation by Rs EPGase. Initial evidence in support of this hypothesis came from experiments showing that water-soluble, heat-stable elicitor was produced in homogenates of castor bean seedlings that were incubated with Rs EPGase.

In a typical experiment, 50 g 55-h-castor bean seedlings were homogenized with 100 ml cold 10 mM sodium phosphate buffer (pH 7.0) in a Waring Blendor for 2 min. To 30 ml of this homogenate was added 1 ml (100 units) of a stock solution of pure Rs EPGase to give a final enzyme concentration of about 3 units/ml. One ml of distilled H2O was added to an additional 30 ml homogenate as a control. Both samples were placed on a rotary shaker for 3.5 h at 30°C. After incubation, the samples were centrifuged at 37,000 g for 15 min. Portions (10 ml) of the aqueous supernatant (S_{20}) from the incubation mixture were heat-treated in a boiling water bath for 20 min to destroy any residual EPGase activity. These solutions were then tested directly in an elicitor bioassay (Table II). Treatment of the homogenate with EPGase produced an elicitor which gave about the same response as a standard amount of EPGase when tested directly. The solution resulting from the homogenate that was not treated with EPGase

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### Table 1. Elicitor Bioassay of Inactivated Rs EPGase

<table>
<thead>
<tr>
<th>Sample</th>
<th>Enzyme Tested</th>
<th>Protein Tested</th>
<th>Radioactivity in Casbene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active EPGase</td>
<td>0.192</td>
<td>0.91</td>
<td>8,342</td>
</tr>
<tr>
<td>Inactivated EPGase</td>
<td>0.190</td>
<td>2.76</td>
<td>6,662</td>
</tr>
<tr>
<td>Inactivated EPGase</td>
<td>0.063</td>
<td>0.92</td>
<td>1,807</td>
</tr>
<tr>
<td>Active and inactivated</td>
<td>0.127</td>
<td>Active: 0.46</td>
<td>5,379</td>
</tr>
<tr>
<td>EPGase</td>
<td></td>
<td>Inactivated: 0.45</td>
<td></td>
</tr>
</tbody>
</table>

* The preparations of the active and inactivated samples of Rs EPGase are described in the text.

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FIG. 2. Elicitor bioassay of active and inactivated Rs EPGase. The data are those from Table I. The height of each bar represents the experimental measurement of the amount of protein, the enzyme activity, or the elicitor activity (dpm in casbene) of each test solution relative to the test solution containing only active enzyme (far left). The heights of the bars for the active EPGase sample have been normalized to 100% for convenience. The solutions tested were as follows: I = active EPGase alone; II = inactivated EPGase containing an amount of enzyme activity equivalent to I; III = inactivated EPGase containing an amount of protein equivalent to I; IV = a mixture containing equal amounts of protein from active EPGase and inactivated EPGase. The protein in all of the tested samples consisted of only active and inactivated Rs EPGase.

Table II. Production of Heat-Stable Elicitor by EPGase Treatment

<table>
<thead>
<tr>
<th>Test Solution</th>
<th>Radioactivity in Casbene$^a$</th>
<th>dpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3 unit Rs EPGase$^b$</td>
<td>1,273</td>
<td></td>
</tr>
<tr>
<td>S$_{37}$ from untreated homogenate</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>S$_{37}$ from EPGase-treated homogenate$^c$</td>
<td>911</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ The elicitor bioassay was carried out as described under “Materials and Methods.” The control value of 714 dpm has been subtracted. The substrate used was $[^{14}C]GPP (2.4 \text{ mCi} / \text{mmol}).$  
$^b$ A positive control consisting of 0.3 unit of Rs EPGase in 10 ml of 10 mm sodium phosphate (pH 7.0).

$^c$ 30 ml of a whole homogenate of 55-h castor bean seedlings was incubated with 100 units of Rs EPGase for 3.5 h at 30°C. 10 ml of heat-treated S$_{37}$ fraction was tested in the elicitor bioassay. See the text for further detail.

gave no elicitation.

Localization of the Source of Heat-Stable Elicitor. These initial studies demonstrated the production of heat-stable elicitor in EPGase-treated castor bean homogenates. It was then of interest to determine which component of the homogenate was the source of the elicitor. A significant portion of the homogenate consisted of insoluble cellular debris and fragmented wall material. It was expected that if the heat-stable elicitor were a pectic digestion product, its precursor should be localized in the insoluble particulate portion of the homogenate containing the wall material.

In a typical experiment, 10 g fresh 55-h seedlings were homogenized in 30 ml of cold 10 mm (pH 6.3) homogenization buffer in a VirTis “23” homogenizer at top speed for 2 min. This homogenate was then separated into P$_{12}$ and S$_{37}$ fractions as described under “Materials and Methods.” Rs EPGase was added to both the S$_{37}$ solution and to a 10% (w/v) suspension of the P$_{12}$ fraction in 10 mm histidine buffer to bring the final enzyme concentration to 1 unit/ml in each. Additional samples of the S$_{37}$ and P$_{12}$ fractions were prepared without added enzyme as controls. The pH of all samples was adjusted to 6.3, and they were placed on a rotary shaker at 120 rpm for incubation at 30°C for 4 h.

At the end of the incubation, the solutions were centrifuged at 37,000g for 15 min. Portions (10 ml) from each supernatant were removed and heat-treated in a boiling water bath for 20 min to destroy any remaining EPGase. These solutions along with a buffer control were tested in an elicitor bioassay (Table III). The untreated S$_{37}$ sample possessed a small amount of elicitor activity, whereas the untreated P$_{12}$ sample had no detectable elicitor activity. Treatment of both the S$_{37}$ and P$_{12}$ fractions with EPGase resulted in distinct increases in heat-stable elicitor, with the increase from the P$_{12}$ fraction being about 5-fold higher than that from the S$_{37}$ fraction. This indicates that the vast majority of the heat-stable elicitor originated from a component(s) of the particulate fraction of castor bean homogenates. A rigorously purified insoluble cell wall fraction was prepared from castor bean seedlings by the procedure of Selvendran (16) and tested as a source of heat-stable elicitor. The uronic acid content of the dry preparation was found to be about 10% by

Table III. Sources of Heat-Stable Elicitor in the Homogenate

<table>
<thead>
<tr>
<th>Sample</th>
<th>Radioactivity in Casbene$^a$ (dpm)</th>
<th>Net Change (dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P$_{12}$</td>
<td>0</td>
<td>11,423</td>
</tr>
<tr>
<td>S$_{37}$</td>
<td>2,388</td>
<td>4,857</td>
</tr>
</tbody>
</table>

$^a$ The elicitor bioassay was carried out as described under “Materials and Methods.” The control value of 5,347 dpm has been subtracted from each of the values.

$^b$ Samples were treated with Rs EPGase as described in the text.

Table IV. Citrus Pectin and Polygalacturonic Acid as Sources of Heat-Stable Elicitor

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration Tested</th>
<th>Radioactivity in Casbene$^a$ (dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pectin</td>
<td>–</td>
<td>2,458</td>
</tr>
<tr>
<td>Pectin</td>
<td>+</td>
<td>12,882</td>
</tr>
<tr>
<td>PGA</td>
<td>–</td>
<td>8,480</td>
</tr>
<tr>
<td>PGA</td>
<td>+</td>
<td>15,305</td>
</tr>
</tbody>
</table>

$^a$ See the text for conditions of treatment with EPGase.

$^b$ The elicitor assay was performed as described under “Materials and Methods.” The control value of 5,384 dpm has been subtracted.

Table V. Elicitor Ability of Other Types of Pectic Fragments

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration Tested</th>
<th>Radioactivity in Casbene$^a$ (dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean cell wall acid hydrolysate$^c$</td>
<td>0.5</td>
<td>18,792$^b$</td>
</tr>
<tr>
<td>PIIF</td>
<td>0.25</td>
<td>8,660$^d$</td>
</tr>
</tbody>
</table>

$^a$ The elicitor bioassay was carried out as described under “Materials and Methods.”

$^b$ The control value of 5,347 dpm has been subtracted.

$^c$ QAE-purified partial acid hydrolysate prepared by Hahn et al. (7).

$^d$ The control value of 5,171 dpm has been subtracted.

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weight. It was shown that treatments of this material with Rs EPGase alone or in combination with active tomato pectinesterase at pH 6.3 did not result in the release of significant amounts of heat-stable elicitor (data not shown). Also, tomato pectinesterase by itself has been shown to have little or no effect on the activity of heat-stable elicitor generated by EPGase treatment of castor bean P12 (data not shown).

Production of Heat-Stable Elicitors from Citrus Pectin and Polygalacturonic Acid by Treatment with Rs EPGase. Citrus pectin and PGA from a commercial source were both tested as possible sources of heat-stable elicitors in the following manner. Solutions of pectin and PGA (0.25%) in 10 mm histidine were prepared and adjusted to pH 6.3. To 10-ml aliquots of the pectin and PGA solutions were added 11.4 and 3.75 units of Rs EPGase, respectively. These solutions were then incubated at 30°C on a rotary shaker for 77 min in the case of pectin and for 97 min in the case of PGA. This procedure resulted in hydrolysis of approximately 8% of the available uronosidic bonds for each sample. Control samples were prepared in an identical fashion, but without the addition of Rs EPGase. Enzymic hydrolysis was stopped by heat-treating the samples at 100°C for 20 min in a boiling water bath. These solutions were then treated directly in an elicitor bioassay (Table IV). Untreated solutions of both pectin and PGA contain some heat-stable elicitor ability by themselves; in both cases, treatment with Rs EPGase results in a large increase in the amount of heat-stable elicitor present.

Elicitor Activity of Other Plant Cell Wall Fragments. Two other plant cell wall fragments have also been tested for activity in the casbene synthetase elicitor assay system. The first is a fraction prepared by Hahn et al. (7) by partial acid hydrolysis of soybean cell walls followed by purification using QAE-Sephadex chromatography. This material is primarily composed of galacturonic acid residues and acts to elicit glyceollin accumulation in soybeans. The second pectic fragment is a sample of PIIF isolated from tomato leaves by Bishop et al. (1). This partially purified material, which also is rich in galacturonic acid residues, has been shown to induce the synthesis of proteinase inhibitors in tomato leaves. Solutions of each of the substances were heat-treated at 100°C for 20 min before testing in the elicitor bioassay (Table V). Both preparations elicited substantial levels of casbene synthetase activity.

Effect of pH on the Production of Heat-Stable Elicitor from Castor Bean P12 Fraction by Rs EPGase Treatment. To test the production of heat-stable elicitor as a function of the pH during incubation with Rs EPGase, a 10% w/v suspension of the particulate fraction from a castor bean homogenate (P12) was prepared in a solution 20 mm in sodium citrate and 20 mm in sodium phosphate. Seven aliquots of this suspension were adjusted to pH 3.20, 4.30, 4.95, 5.65, 6.35, 7.40 and 8.40, respectively, by the addition of an appropriate volume of an HCl or NaOH solution. Rs EPGase was added to each aliquot to a final concentration of 1 unit/ml. A control sample was prepared at pH 6.35 without the addition of the Rs EPGase. The solutions were placed on a rotary shaker at 120 rpm and 30°C for 4 h. After the incubation, each sample was adjusted to pH 6.35 by the addition of acid or base and centrifuged at 37,000g for 15 min. An aliquot of the supernatant fraction from each incubation mixture was heat-treated in a boiling water bath for 20 min. The resulting solutions were tested directly in an elicitor bioassay. In addition, 200-μl aliquots of each supernatant fraction were also assayed for the amounts of reducing ends in order to quantify the extent of enzymic hydrolysis.

Results of the elicitor bioassay are summarized in Figure 3. Included in this graph is the measure of reducing ends enzymically released for each sample. The amount of elicitor produced is minimal at about pH 5, the apparent pH optimum of Rs EPGase with both the castor bean particulate fraction and commercial

![Graph showing variation of the production of heat-stable elicitor by Rs EPGase as a function of pH.](https://example.com/graph.png)
pH may have given extensive conversion of active elicitor fragments to smaller inactive fragments. To test this possibility, solutions containing heat-stable elicitor were incubated with Rs EPGase at its pH optimum. The heat-stable elicitor samples were prepared from both commercial PGA and castor bean P12 by limited digestion with Rs EPGase.

One and a half ml of a solution containing heat-stable elicitor (enzymically produced at pH 6.3 from a 10% w/v suspension of castor bean P12 in a concentrated 6-fold by lyophilization) was added to pH 4.95 with HCl. Ten units of Rs EPGase were then added and the resulting solution was incubated at 30°C for 4 h along with a positive control containing the same heat-stable elicitor solution but without added EPGase. Following incubations, the pH of each solution was adjusted to 6.5, the total volume was brought to 10 ml with distilled H2O, and the solutions were heat-treated at 100°C for 30 min. This set of solutions was tested in an elicitor bioassay.

In a similar manner, two 4-ml aliquots of a solution containing heat-stable elicitor produced by partial enzymic hydrolysis of commercial PGA (0.25% w/v, 8% hydrolysis) were adjusted to pH 4.9. To one was added 4 units of Rs EPGase. Both solutions were incubated for 4 h at 30°C and then adjusted to pH 6.3, brought to 10 ml with distilled H2O, and heat-treated. This set of solutions was then tested directly in an elicitor assay.

The results of both assays are summarized in Table VI. In both cases, the amount of active elicitor is significantly reduced by further digestion with EPGase, thus confirming that substantial inactivation of the enzyme-released heat-stable elicitor can result from prolonged treatment with EPGase at the pH optimum of the enzyme. It seems likely that under the conditions employed, the observed minimum amounts of heat-stable elicitor activity released at the pH optimum of EPGase activity (Fig. 3) results from "over-digestion" of active elicitor to inactive forms. Another possibility is that inhibitors of elicitation had been produced by complete digestion with EPGase. However, a total EPGase digest showed no inhibitory effect on elicitation when mixed with a sample of heat-stable elicitor (unpublished results). This indicates the probable absence of inhibitors of elicitation in the fully digested sample.

**pH Variation of Rs EPGase Elicitation In Vivo.** Experiments to measure the variation of elicitation of castor bean seedlings by Rs EPGase as a function of the pH of the medium were performed in order to examine any possible correlation with the observed variation of the *in vitro* production of heat-stable elicitor as a function of the pH of incubation (Fig. 3). If the heat-stable elicitor is an obligate intermediate in elicitation by EPGase, one might expect to see an *in vivo* pH dependence curve similar to the *in vitro* curve shown in Figure 3. One representative experiment of several performed is described below.

Sterile buffer solutions of approximately equal osmolality were prepared at 5 different pH values ranging between 2.9 and 7.4. A list of the buffers employed appears in the legend for Figure 4. Seedlings were treated with 0.2 units of Rs EPGase added to 10 ml of each buffer in a standard elicitor bioassay. In addition, a control test in which seedlings were incubated with the appropriate buffer, but without added EPGase, was performed at each pH. The final pH of the medium was measured at the conclusion of the period of incubation of the test seedlings with the test solution. The results of the elicitor assays are presented in Figure 4. The exact shape of the profile of elicitor activity of EPGase as a function of pH varies somewhat from that shown in Figure 4 in other experiments of this type. But in all cases, a minimum of elicitation is seen at pH values in the range of 5 to 5.5 with maxima on either side.

Although there are differences in detail, the general shapes of the profiles for the *in vitro* production of heat-stable elicitor activity by Rs EPGase treatment (Fig. 3) and the elicitor activity of Rs EPGase *in vivo* as a function of the pH of the medium (Fig. 4) are similar. In particular, a region of minimal activity is seen in the range of pH 5 to 5.5 with maxima at pH values of approximately 4 and 6.3. There are a number of factors that might influence the interpretation of these results, particularly for the *in vivo* experiments. But the close similarities of the responses provide some support for the idea that the same processes which lead to the release of heat-stable elicitor activity by treatment of cell-free particulate fractions with Rs EPGase *in vitro* are involved in the function of EPGase as an elicitor of casbene synthetase activity *in vivo*.

**Other Properties of the Heat-Stable Elicitor.** Samples of the heat-stable elicitor from castor bean tissue prepared as described under "Materials and Methods" and concentrated 6-fold by lyophilization were tested for acid stability and were assayed for neutral sugar and uronic acid content. Aliquots of the heat-stable elicitor solution (1.5 ml) were added to either 1.5 ml distilled H2O or 1.5 ml of 6 N HCl and were heated at 121°C for 1 h in tightly capped screw cap test tubes. The resulting solutions were then evaporated *in vacuo* to dryness at 60°C, reconstituted in distilled H2O and adjusted to pH 6.5. These solutions were then tested in an elicitor bioassay. Whereas the sample not treated with acid possessed strong elicitor activity, the elicitor activity of the acid-
treated sample was completely destroyed.

The neutral sugar content of the crude elicitor solution was measured using the anthrone assay (20) and the galacturonic acid content was measured using the assay procedure of Blumenkrantz and Asboe-Hansen (2). The results of the assays indicated that galacturonic acid comprised about 70% of the total carbohydrate and that neutral sugar (measured in terms of glucose-equivalents) comprised approximately 30% of the total carbohydrate. It should be noted, however, that the solution which was assayed doublets contained components in addition to heat-stable elicitors which contributed to an unknown extent to this compositional analysis.

DISCUSSION

The evidence summarized in Figure 2 indicates that the catalytic activity of Rs EPGase is necessary for its action as an elicitor of casbane synthetase activity in castor bean seedlings. A close correlation was observed between the amounts of EPGase activity in a mixture of active and inactive enzyme molecules and the level of elicitation it produced. No such relationship was seen between the total concentration of active plus inactive molecules of enzyme in a mixture and its activity as an elicitor. The simplest interpretation of these results is that the catalytic activity, and no other structural feature of Rs EPGase, is responsible for its function as an elicitor in vivo. This interpretation assumes that the covalent modification of the enzyme did not disturb other structural features of the EPGase substantially, an assumption which seems reasonable when one considers the relatively mild and specific modification procedure employed. Earlier results which demonstrated that treatments of Rs EPGase with heat, pronase, and periodate gave parallel losses in enzyme and elicitor activity (12,21) are also consistent with this interpretation.

One way in which the catalytic activity of Rs EPGase could participate in its action as an elicitor would be by causing the breakdown of a normal plant constituent to one or more degradation products which themselves could function as elicitors. Thus, it was of considerable interest to find that incubation of a crude castor bean homogenate with the pure enzyme led to the production of soluble heat-stable elicitor components while none were produced in the control incubation mixture lacking enzyme (Table II). The elicitor activity of the fungal enzyme itself is completely destroyed by the heat treatment employed after the digestion step in these experiments, so the heat-stable elicitor activity observed must have originated from plant material during the incubation.

Rs EPGase acts as an endo-hydrolase with PGA as the substrate (12). It also functions to catalyze the hydrolysis of citrus pectin, although somewhat less efficiently (12). If Rs EPGase functions as an elicitor through its catalytic activity by releasing a heat-stable substance with elicitor activity, it might be anticipated that the heat-stable elicitor is somehow released through the breakdown of pectic components of the plant cell wall. Several lines of experimental observation support the idea that the heat-stable elicitors observed in these experiments consist of pectic cell-wall fragments. (a) The major source of the heat stable elicitor from castor bean homogenates is the particulate fraction rather than the soluble supernatant fraction (Table III). The particulate fraction contains the insoluble cell wall components and presumably includes most of the pectic substances. (b) Heat-stable elicitor is also produced by Rs EPGase action on citrus pectin and PGA. Partial hydrolysis of either of these substances by Rs EPGase results in large increases in elicitor abilities (Table IV). (c) The heat stable elicitors produced from either of these materials in two commercial PGA are both inactivated, although to varying extents, by more exhaustive digestion with pure Rs EPGase (Table VI). Thus, it would appear that the active components in these heat-stable elicitor preparations can serve as substrates for the action of an EPGase. (d) Analysis of the crude heat-stable elicitor fraction released by the action of Rs EPGase on castor bean homogenate indicated that about 70% of the carbohydrate present was galacturonic acid or its methyl ester. It should be emphasized that this is a crude fraction and may contain many components other than elicitor which contribute to its average composition. However, some of the preceding arguments is totally conclusive in itself, but together they support the idea that the heat-stable elicitors produced in castor bean homogenates are degradation products originating from pectic components of the plant cell-wall.

A cell wall fraction from castor bean seedlings purified according to the more rigorous procedures of Selvendran (16) did not serve as a source of heat-stable elicitor when treated with Rs EPGase. Apparently the components which give rise to the heat-stable elicitor on digestion are removed by the more rigorous extraction procedures which involve extensive grinding in a ball mill in the presence of 1% deoxycholate and several washing steps. These wall preparations contained only about 10% by weight of galacturonic acid and its derivatives. Further studies not reported here indicate that the pectic substances which serve as the source of heat stable elicitors on EPGase treatment would probably be solubilized by the procedures employed in the Selvendran method and thus lost from the final wall preparation.

Further support for the pectic nature of the heat-stable elicitors comes from the strong casbane synthetase elicitor activity of fractions rich in polygalacturonides from other systems (Table V). These include the endogenous elicitor of glycooelin prepared from soybean cell walls by partial acid hydrolysis and partially purified by QAE-Sephadex chromatography (7), and a fraction with PIIF activity obtained from tomato leaves (1).

The above results obtained with the R. stolonifer-castor bean seedling interaction can be generalized into a theory of elicitation by fungal pectinolytic enzymes. Because of the presence of the protective polysaccharide wall surrounding plant cells, successful invasion of plant tissues by a fungus is frequently dependent on production by the fungus of enzymes, particularly pectinolytic enzymes, capable of degrading the plant cell wall. The plant, in turn, has evolved a defensive response to the presence of these pectinolytic enzymes. However, instead of developing a recognition system for the enzyme molecules themselves, which may differ widely in structure depending on the fungus of origin, the plant has evolved a recognition system for the pectic fragments of its own cell walls produced by the action of these pectinolytic enzymes. Thus, the pectic fragments could serve as a common intermediate signal indicating the presence of a wide range of potential fungal predators.

Two lines of evidence presented in this paper are consistent with the functioning of a system of this type during the in vivo interaction of R. stolonifer and castor bean seedlings. First, the pH profile of casbane synthetase elicitation by Rs EPGase in vivo (Fig. 4) closely resembles the pH profile of production of heat stable elicitors by the action of Rs EPGase on castor bean homogenates in vitro (Fig. 3). Both show a minimum at pH values near 5 with maxima on either side of this value. This suggests that in vivo elicitation by Rs EPGase requires the intermediate production of heat-stable elicitors. Secondly, the elicitor activity of Rs EPGase has been shown to closely parallel the level of enzymic activity associated with the enzyme irrespective of the total level of active plus inactive EPGase molecules present. This is consistent with an hypothesis requiring the catalytic activity of the enzyme for the production of pectic fragments as intermediates in elicitation and inconsistent with the possibility of direct recognition of some other structural feature of the enzyme molecule by the plant. No evidence has been obtained for any macromolecular elicitor of casbane synthetase from R. stolonifer cultures other than EPGase.

It seems likely from the observations to date that the heat-stable elicitor will prove not to be a single molecular species, but rather a family of pectic fragments that possess some required set of
molecular characteristics in common. Work in our laboratory is currently underway to help elucidate the structural features neces-
sary for activity in this class of heat-stable elicitors.

The possible importance of fungal enzymes as elicitors and the in vivo importance of pectic fragments in the elicitation process have not been recognized until recently. A contributing factor to this has doubtless been the fact that the techniques for the extraction and purification of fungal elicitors have not generally been ones which would preserve enzyme activity (25). Hargreaves and Bailey (8) have described the formation of heat-stable "constitutive elicitors" in living plant tissues when brought into contact with dead tissues. The chemical nature of these elicitors and their relationship to the heat-stable elicitors described in this work are not known. The recent demonstration by Hahn et al. (7) of galacturonide-rich "endogenous elicitors" of glyceollin in hot water extracts and partial-polyuronide-rich cell walls of soybeans and other plants is also of direct relevance to this hypothesis as mentioned earlier.

Even though the in vivo significance of pectic elicitors in a defense response has not been well documented in other plant-fungus systems to date, we believe there are a number of reasons for considering the possibility that this is a widespread mechanism involved in general resistance. Further arguments in support of this view are presented in a recent review article (25).

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