Proteins from Plant Cell Walls Inhibit Polygalacturonases Secreted by Plant Pathogens

(Part-host-pathogen interactions/fungi/Colletotrichum lindemuthianum/Fusarium oxysporum/Sclerotium rolfsii/agglutinins)

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ABSTRACT Proteins extracted from the cell walls of Red Kidney bean hypocotyls, tomato stems, and suspension-cultured sycamore cells can completely inhibit the activity of the polygalacturonases (polygalacturonidase hydrolases, EC 3.2.1.15) secreted by the fungal plant pathogens Colletotrichum lindemuthianum, Fusarium oxysporum, and Sclerotium rolfsii. The inhibitor of the C. lindemuthianum polygalacturonase, purified 560-fold from bean hypocotyl extracts, is 40 times as effective an inhibitor of the C. lindemuthianum polygalacturonase as of the F. oxysporum polygalacturonase, and does not demonstrably inhibit the S. rolfsii polygalacturonase. A crude hypocotyl extract that completely inhibits the three polygalacturonases does not inhibit C. lindemuthianum-secreted cellulase, xylanase, α-galactosidase, α-arabinofuranosidase, or α-galacturonidase. The purified bean hypocotyl protein combines with the C. lindemuthianum polygalacturonase to form a complex with a dissociation constant of $2 \times 10^{-8}$ M or less. The physical properties of these inhibitors are similar to those of phytohemagglutinins and of the plant glycoproteins capable of agglutinating transformed animal cells.

Hemagglutinins have been found to be widespread in the seeds of higher plants, and have been studied, especially, in the seeds of legumes (1-3). More recently, it has been demonstrated that similar glycoproteins agglutinate transformed animal cells (4, 5). The plant agglutinins appear to function by recognizing glycosides present in cell-surface polymers of the animal cells.

What are these agglutinins doing in plants? One possibility is that these multifunctional glycoproteins have evolved in response to the presence of plant pathogens. The agglutinins may act to defend the plant against infection by, in some manner, controlling the growth of plant pathogens. If this is true, the agglutinins may be analogous to animal antibodies.

Plant pathogens all secrete enzymes capable of degrading the polysaccharides of plant cell walls (6). When pathogens are grown on isolated cell walls as the sole carbon source, the pathogens secrete polysaccharide-degrading enzymes into the medium. The fungal pathogen Colletotrichum lindemuthianum secretes different enzymes in a temporal sequence with regard to culture age. The first enzyme secreted is an endopolygalacturonase (7). This enzyme has been purified to what is believed to be a homogeneous state (English, P. D., A. Maglothin, and P. Albersheim, in preparation), and the pure enzyme retains the ability to remove most of the galacturonic acid from the isolated cell walls of a number of plants. More recently (Jones, T. M., A. Anderson, and P. Albersheim, in preparation), it has been demonstrated that the tomato pathogen, Fusarium oxysporum f. sp. lycopersici, also secretes wall-degrading enzymes into the culture medium in a sequential manner; the first enzyme to be secreted is also one that degrades polygalacturonic acid. We report here that the cell walls of Red Kidney bean hypocotyls, of tomato stems, and of sycamore cells grown in suspension culture contain proteins able to inhibit, completely and specifically, the activities of the early polygalacturonases secreted by C. lindemuthianum and F. oxysporum, and also the activity of a polygalacturonase secreted by Sclerotium rolfsii. Other polysaccharide-degrading enzymes secreted by these pathogens are unaffected by extracts containing the inhibitors.

MATERIALS AND METHODS

Red Kidney bean (Phaseolus vulgaris) seedlings were grown as described (7). Tomatoes (Lycopersicon esculentum) “Clark’s Special” and “Jefferson” were grown in sand for 6 weeks, and cell walls were prepared from the stems as described (7).

The endopolygalacturonase secreted by the alpha strain of C. lindemuthianum was induced by growing the fungus in shake culture on minimal salts media containing 1% citrus pectin (the gift of Sunkist Growers, Inc.) (7). A solution containing 0.1 μg of 100-fold purified endopolygalacturonase hydrolyzes, during incubation for 1 hr at 30°C, 1.25 μmol of the galacturonide linkages of 0.1% polygalacturonic acid in 1 ml of 50 mM sodium acetate (pH 5.2). S. rolfsii was grown and the polygalacturonase prepared by the methods described (8).

F. oxysporum f. sp. lycopersici was cultured according to published methods (9), but extracellular enzymes were produced by growing the fungus on salts media containing 1% isolated tomato cell walls, rather than 1% pectin. Xylanase and cellulase, partially purified from the extracellular fluid of C. lindemuthianum cultures grown on xylan and cellulose, respectively, were provided by Kenneth Keegstra.

Assays

Polygalacturonase, cellulase, and xylanase were assayed by the method described (10). The substrate for the polygalacturonase consisted of 0.1% (w/v) citrus pectin that had been de-esterified (pH 12, 0°C, 2 hr) and reduced with NaBH$_4$ (10 mg/liter, pH 12, 0°C, 1 hr). The activities of xylanase (EC 3.2.1.8) and of cellulase (EC 3.2.1.4) were measured in 0.1% solutions of xylan (Koch-Light Laboratories, Ltd.) and of CM-cellulose (Hercules, Inc.), respectively. The

Part III of Host-Pathogen Interactions. Part II is ref. 7.
polygalacturonase was also measured viscometrically (7). The activities of highly purified C. lindemuthianum α-galactosidase (EC 3.2.1.22) and α-arabinofuranosidase (the gifts of Kenneth Keegstra) were measured by their ability to hydrolyze the respective p-nitrophenyl glycosides (7).

Endopolygalacturonase inhibitor activity was normally determined by the use of sufficient inhibitor to reduce the rate of polygalacturonic acid degradation by 50%. One unit of inhibitor is defined as the amount that prevents the formation of 0.7 μmol of galacturonic acid-reducing equivalents in 1 hr at 30°C when the reaction is performed in 50 mM sodium acetate (pH 5.2), and in a total volume of 1 ml. This solution usually contains 800 μg of polygalacturonic acid (equivalent to 4.5 μmol of galacturonic acid), and an amount of polygalacturonase that produces 1.4 μmol of galacturonic acid-reducing equivalents in the absence of inhibitor.

Proteins were measured by the method of Lowry et al. (11). All experiments were performed at 2°C unless otherwise specified.

**Standard buffers**

The standard Buffer used in these studies in 50 mM sodium acetate (pH 5.2). This solution is referred to in this manuscript as "Buffer." Buffer that contains 1 mM each of CaCl₂, MnCl₂, and MgCl₂ is referred to as "Buffer plus Metals."

**Extraction and purification of inhibitor from Red Kidney bean hypocotyls**

The hypocotyl protein that inhibits the C. lindemuthianum polygalacturonase was extracted as follows. 500 g of 8-day-old hypocotyls was chopped into 2- to 4-mm segments with a razor blade. The segments and 1 liter of 0.5 M potassium phosphate (pH 7) were homogenized for 60 sec in a Waring Blender. The resulting suspension was filtered without suction through a coarse sintered-glass filter. The residue was homogenized twice more, first with 750 ml and then with 500 ml of the phosphate buffer. The extracts were combined (2280 ml) and adjusted to pH 4.6 with 4 N HCl. After 30 min, the solution was readjusted to pH 7 with 4 N NaOH and the insoluble material was removed by centrifugation for 30 min at 13,000 × g.

The supernatant fraction was adjusted to 20% of saturation with (NH₄)₂SO₄ and stirred for 30 min. The resulting suspension was centrifuged at 13,000 × g for 20 min and the pellet was discarded. Further ammonium sulfate treatment yielded the proteins insoluble in 20–40% and 40–70% (NH₄)₂SO₄. The 40–70% pellet was dissolved in 80 ml of Buffer and dialyzed for 16 hr against 5 mM sodium acetate (pH 5.2). The precipitate formed was removed by centrifugation for 5 min at 48,000 × g. The insoluble material was washed with 5 ml of 5 mM NaOAc (pH 5.2), and the wash was added to the supernatant fraction. This soluble portion was passed through a DEAE-cellulose column (1.3 × 12 cm) that had previously been equilibrated with the 5 mM Buffer. The material that passed through the column and 150 ml of 5 mM Buffer used to wash the column were combined.

The eluate from the DEAE-cellulose column, 200 ml, was equilibrated with Buffer plus Metals and passed through a Sephadex G-25–300 (Sigma Chemical, Lot 48B-1860) column (1.4 × 14 cm). The Sephadex was not used for its ability to size molecules but as a specific adsorbent of the polygalacturonase inhibitor. The Sephadex column was washed with 200 ml of Buffer plus Metals, then with 175 ml of the same solution containing 0.1 M glucose, and again with 150 ml of Buffer plus Metals. Up to this point, all of the material passing through the Sephadex G-25–300 column was discarded. Finally, the column was subjected to gradient elution by mixing in linear fashion 200 ml of Buffer plus Metals and 200 ml of Buffer plus Metals containing 100 mM NaCl. The protein and inhibitor concentrations in each of the 12-ml fractions collected from this gradient elution were assayed (Fig. 1). Fractions 90–99 were combined and dialyzed against Buffer plus Metals. To concentrate the inhibitor, we passed the combined fractions through a second Sephadex G-25–300 column (1.6 × 6 cm). The solution that passed through this column was discarded. The column was then eluted with Buffer plus Metals containing 300 mM NaCl. 1-ml fractions were collected and assayed for inhibitor. Fractions 6–11 were combined and 4.8 ml of this solution was chromatographed on a Bio Gel P-100 column (2.2 × 89 cm, void volume 96 ml) by elution with Buffer plus Metals containing 100 mM NaCl. The protein and inhibitor concentrations in each of the 12-ml fractions were assayed. Fractions 12–14 were combined and the inhibitor was concen-
trated on a Sephadex G-25-300 column (1.6 × 6 cm) as described above. This concentrate (5.0 ml) was applied to a Bio Gel P-150 column (1.4 × 44 cm, void volume 26 ml). The column was eluted with Buffer plus Metals containing 100 mM NaCl. The protein and inhibitor concentrations in each of the 6.2-ml fractions were assayed (Fig. 2).

Polygalacturonase inhibitors from sycamore cells and tomato stems

Cells of sycamore (Acer pseudoplatanus) cultured in suspension were collected on a coarse sintered-glass filter and washed three times with 10 volumes of 10 mM potassium phosphate (pH 7). We extracted the inhibitors by suspending the washed cells in 3 volumes of 500 mM potassium phosphate (pH 7), filtering under reduced pressure, suspending the cells again in 3 volumes of the 500 mM phosphate buffer, combining the extracts, and adding (NH₄)₂SO₄ to 70% of saturation. The resulting precipitate was collected by centrifugation at 46,000 × g for 20 min. The pellet was suspended in Buffer; the soluble portion contained the polygalacturonase inhibitors.

6-week-old tomato stems were chopped with a razor blade into 2- to 4-mm segments. The segments and 3 volumes of 100 mM potassium phosphate (pH 7) were homogenized in a Waring Blender for 60 sec. The supernatant solution was removed by filtration through a coarse sintered-glass filter and discarded. The residue was homogenized with 3 volumes of the 100 mM phosphate buffer, and again the supernatant fraction was discarded. We ground the residue with 3 volumes of 500 mM potassium phosphate (pH 7), collected the solution by filtration, re-extracted the residue with 3 volumes of the 500 mM phosphate buffer, and combined the extracts. (NH₄)₂SO₄ was added to 70% of saturation, and the precipitate was collected by centrifugation for 5 min at 48,000 × g. The pellet was suspended in Buffer; the soluble portion contained the polygalacturonase inhibitors.

RESULTS

Table 1 shows the course of purification of the Red Kidney bean hypocotyl protein that inhibits the polygalacturonase secreted by C. lindemuthianum. The inhibitor binds to carboxyl anion exchangers, but the inhibitor activity recovered from such columns was sufficiently low to exclude this procedure from the purification scheme.

Prior incubation of the inhibitor with the polygalacturonase is not required for effective inhibition. This was demonstrated by comparison of the ability of the purified Red Kidney hypocotyl protein with that of the Nelson-Somogyi copper reagent to terminate the hydrolysis of polygalacturonic acid catalyzed by the C. lindemuthianum polygalacturonase. Addition of the inhibitor stopped the enzymic reaction at least as quickly as addition of the alkaline copper reagent.

The degree of polygalacturonase inhibition depends on the amount of inhibitor added (Fig. 3). The reaction rate for polygalacturonase depends on the substrate concentration (Fig. 4); polygalacturonic acid concentrations above 0.06% result in substrate inhibition. Nevertheless, the substrate concentration has little effect on the degree of inhibition resulting from the presence of the Red Kidney bean inhibitor (Fig. 4).

Red Kidney bean hypocotyl inhibitor is relatively stable to heat. Heating a solution of the polygalacturonase inhibitor for 210 min at 50°C destroys only about 18% of its activity; at 55°C, about 77% is lost in the same period. Essentially all of the activity is destroyed after 15 min at 70°C.

The 0–70% (NH₄)₂SO₄ precipitates of the extracts of the cell walls of tomato stems, sycamore cells, and bean hypocotyls are each capable of inhibiting completely the polygalacturonases secreted by C. lindemuthianum F. oxysporum, and S. rolfsii (Table 2), although different amounts of protein are required for each of the extracts to inhibit the three polygalacturonases. This differential ability of the different plant extracts to inhibit the three polygalacturonases suggests that the three plant species
have inhibitors with different properties and, furthermore, that there may be several inhibitors within a given plant.

This suggestion receives support from the observation that purified inhibitor from Red Kidney bean hypocotyls is a poor inhibitor of the *F. oxysporum* and *S. rolfsii* polygalacturonases, whereas the relatively impure (NH₄)₂SO₄ precipitate has rather similar effects on all three fungal enzymes. Further, the inhibitors of the *C. lindemuthianum* and *F. oxysporum* enzymes are extracted from the hypocotyl cell walls of Red Kidney bean by 50 mM sodium acetate (pH 5.2) containing 300 mM NaCl whereas the *S. rolfsii* polygalacturonase inhibitor is not. This inhibitor is extracted in 500 mM potassium phosphate (pH 7) (data not shown).

The *Red Kidney bean hypocotyl extracts do not inhibit other polysaccharide-degrading enzymes.* In contrast to its action on the endopolygalacturonases, the 0–70% (NH₄)₂SO₄ precipitate of the Red Kidney bean hypocotyl extracts does not inhibit purified preparations of α-arabinosidase, α-galactosidase, carboxymethylcellulase, xylanase, and exopolygalacturonidase, all of which are secreted by *C. lindemuthianum* during growth on isolated cell walls, in contrast to the action on the endopolygalacturonases.

**DISCUSSION**

The *C. lindemuthianum* polygalacturonase inhibitor isolated from Red Kidney bean hypocotyls has several properties which suggest that it is one of the glycoproteins commonly referred to as phytoagglutinins. The heat stability of the inhibitor is similar to that of the phytoagglutinins (e.g., refs. 5, 12). The approximate molecular weight of this protein (50,000 from its elution characteristics on Bio Gel P-100 and P-150) agrees with those for a number of phytoagglutinins (12–14). In common with the phytoagglutinins, the polygalacturonase inhibitor fails to bind to DEAE-cellulose but binds to carboxymethyl cation-exchange columns (12, 14–17). And, like a number of phytoagglutinins, the inhibitor binds to Sephadex G-25–300 (13, 14, 16, 18, 19). We have also observed that the inhibitor from hypocotyls has an affinity for Agarose-0.5 m.

The presence in Red Kidney beans of agglutinins of erythrocytes has been shown repeatedly (15, 17, 20–22). Tunis (23) has demonstrated that the seeds contain a protein which agglutinates transformed animal cells but not erythrocytes. Thus, Red Kidney beans have agglutinins that do not agglutinate erythrocytes, and these perhaps include the polygalacturonase inhibitors.

The suggestion that the polygalacturonase inhibitors are phytoagglutinins would be more firmly established if a hapten for the inhibition reaction could be identified. Many carbohydrates were tested and found not to prevent the protein from Red Kidney bean hypocotyls from inhibiting the *C. lindemuthianum* polygalacturonase. The failure to find such an antagonist may be due to binding of the inhibitor to a complex oligosaccharide on the surface of the enzyme rather than to a simple sugar (24). A hapten that represents only a portion of the binding site would have to be present in very high concentration to counteract the strong affinity between enzyme and inhibitor. In a 1-mI reaction mixture, less than 10 pmol of the inhibitor completely inactivates about 0.3 pmol of the polygalacturonase; from such data we have calculated a maximum dissociation constant of 2 × 10⁻¹⁰ M.

What is the significance of the inactivation of endopolygalacturonases by plant proteins? Extracts of some plants have previously been shown to inhibit pectinase activity (25, 26), although, with one possible exception (27), this inhibition has been attributed to phenolic compounds. These compounds are general enzyme inhibitors; they lack specificity for pectinase. However, plant proteins have been isolated that are more specific inhibitors of degradative enzymes. These include the soybean trypsin inhibitors (28, 29) and the invertase inhibitors from potatoes and beets (30).

Our results show that plant cell walls contain proteins that inhibit pathogen-secreted endopolygalacturonases but not

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**Table 1. Purification of the protein from Red Kidney bean hypocotyls that inhibits the *C. lindemuthianum* polygalacturonase**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Total units*</th>
<th>Yield (%)</th>
<th>μg protein/ml</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude (dialyzed)</td>
<td>2230</td>
<td>27,000</td>
<td>100</td>
<td>5,400</td>
<td>45</td>
</tr>
<tr>
<td>pH 4.6 supernatant</td>
<td>2500</td>
<td>32,000</td>
<td>120</td>
<td>3,600</td>
<td>28</td>
</tr>
<tr>
<td>40–70% (NH₄)₂SO₄</td>
<td>80</td>
<td>17,350</td>
<td>64</td>
<td>6,000</td>
<td>28</td>
</tr>
<tr>
<td>5 mM acetate dialysis</td>
<td>76</td>
<td>16,900</td>
<td>63</td>
<td>2,400</td>
<td>11</td>
</tr>
<tr>
<td>DEAE-cellulose eluate</td>
<td>200</td>
<td>15,600</td>
<td>58</td>
<td>470</td>
<td>6</td>
</tr>
<tr>
<td>Sephadex-gradient</td>
<td>85</td>
<td>7,000</td>
<td>26</td>
<td>31</td>
<td>0.37</td>
</tr>
<tr>
<td>Sephadex concentrate</td>
<td>5.5</td>
<td>5,500</td>
<td>20</td>
<td>350</td>
<td>0.35</td>
</tr>
<tr>
<td>Bio Gel P-100</td>
<td>48</td>
<td>3,694</td>
<td>14</td>
<td>9</td>
<td>0.120</td>
</tr>
<tr>
<td>Bio Gel P-150</td>
<td>20</td>
<td>2,000</td>
<td>7</td>
<td>8</td>
<td>0.08</td>
</tr>
</tbody>
</table>

* See Materials and Methods for assay conditions and definition of units.

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**Table 2. Amount of protein required for 50% inhibition of three polygalacturonases (relative values)**

<table>
<thead>
<tr>
<th>Inhibitor source</th>
<th>Polygalacturonase source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>C. lindemuthianum</em></td>
</tr>
<tr>
<td></td>
<td><em>oxysporum</em></td>
</tr>
<tr>
<td></td>
<td><em>S. rolfsii</em></td>
</tr>
<tr>
<td>&quot;Clark's Special&quot; tomato stems</td>
<td>1*</td>
</tr>
<tr>
<td>Jefferson tomato stems</td>
<td>1*</td>
</tr>
<tr>
<td>Sycamore cells</td>
<td>4*</td>
</tr>
<tr>
<td>Red Kidney bean hypocotyls (NH₄)₂SO₄ ppt.</td>
<td>1†</td>
</tr>
<tr>
<td>&quot;Purified&quot;</td>
<td>1§</td>
</tr>
</tbody>
</table>

* 14 μg of protein; † 7 μg of protein; § 28 μg of protein; ¶ 0.1 μg of protein; ‡ No measurable activity with 10 μg of protein.
several other enzymes, and distinguish between polygalacturonases secreted by different species of pathogenic fungi. This suggests that such inhibitors participate in disease resistance.

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