Host-Pathogen Interactions

VI. A SINGLE PLANT PROTEIN EFFICIENTLY INHIBITS ENDOPOLYGALACTURONASES SECRETED BY COLLETOTRICHUM LINDEMUTHIANUM AND ASPERGILLUS NIGER

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ABSTRACT

Endopolygalacturonases have been purified from the extracellular enzymes of Colletotrichum lindemuthianum and Aspergillus niger. A protein, purified from Red Kidney (Phaseolus vulgaris) beans for its ability to inhibit the endopolygalacturonase secreted by C. lindemuthianum, inhibits the A. niger endopolygalacturonase almost as efficiently as it inhibits the C. lindemuthianum enzyme.

Plant pathogens have the ability to secrete enzymes capable of degrading the polysaccharides of plant cell walls (1). Of these enzymes, those that degrade pectin are believed to be of key importance in pathogenesis. The pectic-degrading enzymes secreted by a number of pathogens cause maceration of plant tissue, loss in electrolytes, and cell death (4, 6, 10, 14). Also, pectic-degrading enzymes have been detected in lesion areas of infected plant tissue (2, 5, 9, 15). Furthermore, an endopolygalacturonase secreted by Colletotrichum lindemuthianum is the only polysaccharide-degrading enzyme of several that have been tested, which will initiate degradation of isolated plant cell walls (8). Pretreatment of isolated cell walls by endopolygalacturonase is a requirement for effective degradation of the walls by other polysaccharide-degrading enzymes (8, 12). A polygalacturonase is the first of a series of polysaccharide-degrading enzymes to be secreted into the culture medium when either C. lindemuthianum or Fusarium oxysporum f. sp. lycopersici is grown on isolated plant cell walls [7, 11]. These early polygalacturonases are completely inhibited by proteins extracted from a variety of plant cell walls including those of Red Kidney hypocotyls (1). The activities of the polygalacturonases secreted by the only other fungi that we have examined, Sclerotium rolfsii (1), and, as reported here, Aspergillus niger, are also inhibited by extracts from Red Kidney bean hypocotyls. This indicates that plants have proteins capable of inhibiting the endopolygalacturonases secreted by a wide variety of fungal pathogens. It seemed unlikely, however, that a unique inhibitor protein would be required for each fungal endopolygalacturonase, since plants would then have to possess an unrealistically large number of different polygalacturonase inhibitors.

The properties of a previously characterized inhibitor of a fungal endopolygalacturonase suggest that a single plant pro-

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tein may inhibit the endopolygalacturonases secreted by more than one pathogen (1). This inhibitor was isolated from Red Kidney bean hypocotyls and was purified 560-fold for its ability to inhibit the endopolygalacturonase secreted by C. lindemuthianum. Although this purified inhibitor has no activity against the polygalacturonase secreted by S. rolfsii, the inhibitor does possess some ability to inhibit the polygalacturonase secreted by F. oxysporum; the purified inhibitor is 40 times more effective against the C. lindemuthianum endopolygalacturonase than against the F. oxysporum enzyme.

The results reported in this paper demonstrate that the highly purified inhibitor of the endopolygalacturonase secreted by C. lindemuthianum can inhibit, with almost equal efficiency, an endopolygalacturonase secreted by A. niger.

MATERIALS AND METHODS

Pectinol R-10, a commercial preparation of enzymes synthesized by A. niger, was the gift of Rohm and Haas Company. The C. lindemuthianum endopolygalacturonase was purified as described (8) from culture filtrates of the fungus grown on citrus pectin. The inhibitor of the C. lindemuthianum endopolygalacturonase was extracted from the cell walls of 8-day-old Red Kidney (Phaseolus vulgaris) bean hypocotyls and purified as described (1).

Assays. Polygalacturonase activity was assayed as described (1). One unit of the polygalacturonase is defined as that amount of enzyme which releases 1.4 μmoles 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 μmoles of galacturonic acid). The polygalacturonic acid was the gift of Sunkist Growers, Inc., Ontario, California. One unit of the endopolygalacturonase inhibitor is defined as that amount which reduces the activity of one unit of the C. lindemuthianum endopolygalacturonase by 50% when assayed under these conditions.

Endopolygalacturonase activity was also measured viscometrically as described (8). The substrate consisted of 0.84% (w/v) citrus pectin in 50 mm sodium acetate, pH 5.2, (the gift of Sunkist Growers, Inc.), that had been reduced using NaN3H, (100 μg/ml, pH 10.0, 0 C, 1 hr). Polygalacturonic acid lyase activity was assayed as described (3). Protein was measured by the method of Lowry et al. (13).

RESULTS AND DISCUSSION

Fractionation of the Polygalacturonases from Pectinol R-10 on DEAE2-Sephadex. Pectinol R-10 contains several physi-

2 Abbreviation: DEAE: diethylaminoethyl.
cally separable polygalacturonases; one of these enzymes was extensively purified and a second was partially purified. An extract of Pectinol R-10 was prepared by suspending 450 mg of the dry powder in 15 ml of 10 mM sodium acetate, pH 5.2. The insoluble material was removed by centrifugation for 5 min at 13,000g. The supernatant liquid was applied to a DEAE-Sephadex A-25 column (1.4 × 21 cm) that had been equilibrated with 10 mM sodium acetate, pH 5.2. The column was washed with 200 ml of 10 mM sodium acetate, pH 5.2, followed by 400 ml of the same buffer containing 150 mM NaCl. The column was then eluted with a linear gradient formed from 300 ml of 10 mM acetate buffer containing 150 mM NaCl and 300 ml of 10 mM acetate buffer containing 400 mM NaCl. Five-ml fractions were collected in tubes containing 100 μl of an 0.5% (w/v) solution of bovine plasma albumin. The albumin was present to stabilize the polygalacturonase. Those fractions containing NaCl were dialyzed for 18 hr against 10 mM sodium acetate, pH 5.2. Each fraction was assayed for polygalacturonase activity and those fractions which contained this activity were reassayed in the presence of two units of inhibitor (Fig. 1). The inhibitor preparation used for this assay had been purified 120-fold from a crude extract of Red Kidney bean hypocotyls by following the procedures described as far as the gradient elution from Sephadex G-25-300 (1).

Fractionation of the A. niger protein on DEAE-Sephadex yields three distinct polygalacturonases, each of which can be completely inhibited by the protein purified from Red Kidney bean hypocotyls for its ability to inhibit the C. lindemuthianum endopolygalacturonase. Aspergillus niger polygalacturonase I does not bind to the DEAE-Sephadex column in 10 mM acetate buffer, pH 5.2. Polygalacturonase II is eluted from the DEAE-Sephadex column in the 150 mM NaCl wash, and polygalacturonase III is eluted in the NaCl gradient. Inhibition of polygalacturonase I requires an amount of inhibitor 15-fold greater than that required to inhibit an equivalent activity of C. lindemuthianum endopolygalacturonase. Since only two units of inhibitor activity were used during the assay of the fractions from this column presented in Figure 1, no inhibition of those fractions containing polygalacturonase I was expected and no inhibition of these fractions was observed. Polygalacturonase II was not examined further. However, the Red Kidney protein was very effective as an inhibitor of polygalacturonase III. This enzyme was further purified as described below.

**Chromatography of A. niger Polygalacturonase III on Bio-Gel P-150.** The DEAE-Sephadex fractions containing polygalacturonase III (Nos. 195–215) were combined and concentrated to 6 ml using an Amicon ultrafilter with a PSWP Pellicon membrane under 40 p.s.i. of nitrogen. Two and one-half ml of this concentrated polygalacturonase preparation were applied to a Bio-Gel P-150 column (1.4 × 91 cm) and

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**Fig. 1.** DEAE-Sephadex A-25 chromatography of an extract from Pectinol R-10. Polygalacturonase was assayed in the absence (○) and in the presence (●) of two units of inhibitor. See text for conditions of assay and for the details of the chromatography. The three peaks of endopolygalacturonase activity described in the text are denoted by I, II, and III.

**Fig. 2.** Bio-Gel P-150 chromatography of polygalacturonase III. Polygalacturonase was assayed in the absence (○) and in the presence (●) of two units of inhibitor. See text for conditions of assay and for the details of the chromatography.

**Fig. 3.** A comparison of the ability of the A. niger polygalacturonase III to hydrolyze the glycosidic linkages of polygalacturonic acid with the ability of this enzyme to reduce the viscose properties of this polymer. See text for experimental details.
the column eluted with 50 mM sodium acetate, pH 5.2. Four-mL fractions were collected in tubes containing 200 μl of 0.5% (w/v) bovine plasma albumin. The fractions were assayed for polygalacturonase activity in the absence and in the presence of two units of inhibitor (Fig. 2). The fractions containing polygalacturonase activity (Nos. 17–25) were combined and concentrated to 4.0 ml by ultrafiltration as described above. This concentrated polygalacturonase preparation was assayed in the presence and in the absence of a more highly purified preparation of inhibitor. This inhibitor preparation had been purified 190-fold from the crude plant extract by following the full procedure described (1).

Properties of Polygalacturonase III. By using the more highly purified inhibitor preparation, 370 μg of protein are required for one unit of inhibition of the A. niger polygalacturonase III. In comparison, 240 μg of inhibitor protein are required for one unit of inhibition of the C. lindemuthianum endopolygalacturonase. Thus, the purified protein isolated from Red Kidney bean hypocotyls is only 1.5 times more effective an inhibitor of the endopolygalacturonase secreted by C. lindemuthianum than it is of the polygalacturonase secreted by A. niger.

Like the C. lindemuthianum enzyme (8), A. niger polygalacturonase III hydrolyzes polygalacturonic acid in an endo- rather than an exo- fashion (Fig. 3). This was shown by observing that the A. niger enzyme reduces the viscosity of a solution of polygalacturonic acid by 50% while hydrolyzing only 0.3% of the glycosidic bonds. Also, like the C. lindemuthianum enzyme (8), A. niger endopolygalacturonase III cleaves the polygalacturonic acid chains by a hydrolytic mechanism rather than by an elimination mechanism. This was demonstrated by following the absorbance at 235 nm during the degradation of polygalacturonic acid by the purified A. niger endopolygalacturonase (3). While the enzyme was producing 1.13 μmoles per ml of polygalacturonic acid-reducing equivalents, there was no measurable increase in absorbance at 235 nm. If the endopolygalacturonase had been a lyase, an increase in absorbance of 5.2 would have been expected.

The elution volume of endopolygalacturonase III from the Bio-Gel P-150 column gives an estimate of the molecular weight of the enzyme. This endopolygalacturonase elutes at 86 ml, whereas lysozyme (mol wt = 14,400), which was used as a standard, elutes at 109 ml (8). By using this information and assuming the enzyme behaves as a sphere, the calculated molecular weight of the endopolygalacturonase is approximately 31,000.

In summary, the importance of pectic-degrading enzymes in plant diseases caused by fungi (see Introduction) stimulated our interest in the proteins associated with plant cell walls that could inhibit polygalacturonases secreted by fungal pathogens. Crude extracts of Red Kidney bean hypocotyls can completely inhibit the polygalacturonases secreted by the three plant pathogens, C. lindemuthianum, S. rolfsii, and F. oxysporum. However, the protein from Red Kidney beans purified for its ability to inhibit the C. lindemuthianum endopolygalacturonase is 40 times more effective an inhibitor of this enzyme than of the polygalacturonase secreted by F. oxysporum. The purified protein has no ability to inhibit the S. rolfsii polygalacturonase (1). This raised the question as to whether a unique inhibitor protein exists for each polygalacturonase secreted by each race of every fungal strain. In the present paper, we demonstrate that a single plant protein can inhibit effectively the endopolygalacturonases secreted by two otherwise unrelated fungi, C. lindemuthianum and A. niger. In our studies we have attempted to inhibit the polygalacturonases secreted by a total of four fungi and have found that the polygalacturonases from two of these fungi are inhibited by a single plant protein. Since there are many fungal polygalacturonases, the possibility that we have selected by chance the only two that are inhibited effectively by this plant protein is remote. This finding suggests that the number of polygalacturonase inhibitor proteins a plant possesses is probably not a very large number, and yet, plants are probably able to inhibit the endopolygalacturonases secreted by a wide variety of plant pathogens.

LITERATURE CITED