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Host-Pathogen Interactions

VII. PLANT PATHOGENS SECRETE PROTEINS WHICH INHIBIT ENZYMES OF THE HOST CAPABLE OF ATTACKING THE PATHOGEN[1]

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ABSTRACT

The results presented demonstrate that microbial pathogens of plants have the ability to secrete proteins which effectively inhibit an enzyme synthesized by the host; an enzyme whose substrate is a constituent of the cell wall of the pathogen. The system in which this was discovered is the anthracnose-causing fungal pathogen (*Colletotrichum lindemuthianum*) and its host, the French bean (*Phaseolus vulgaris*). An endo-β-1,3-glucanase present in the bean leaves is specifically inhibited by a protein secreted by *C. lindemuthianum*. The cell walls of *C. lindemuthianum* are shown to be composed largely of a 1,3-glucan.

Microbial pathogens of plants secrete enzymes capable of degrading the polysaccharides of plant cell walls (4). When pathogens are grown with isolated cell walls as the sole carbon source, polysaccharide-degrading enzymes are secreted into the medium. Several fungal pathogens secrete degradative enzymes in a temporal sequence with regard to culture age. The first degradative enzyme secreted by three fungal pathogens is an endopolygalactouronase (9, 13, J. Mullen and D. W. Bateman, personal communication). An apparently homogeneous preparation of the endopolygalacturonase secreted by the fungal pathogen *Colletotrichum lindemuthianum* retains the ability to remove most of the galacturonosyl residues from the isolated cell walls of a number of plants (10). This endopolygalacturonase is the only enzyme, of several polysaccharide-degrading enzymes studied, which could initiate degradation of isolated cell walls. Therefore, it was of considerable interest to find that a variety of dicotyledonous plants contain proteins, associated with their cell walls, that are able to inactivate the endopolygalacturonases that are among the first degradative enzymes secreted by the fungal pathogens during culture (3).

Just as pathogens can secrete enzymes capable of degrading plant cell walls, plants can synthesize enzymes capable of degrading the cell walls of their pathogens. Abeles and Forrence (2) have demonstrated that beans (*Phaseolus vulgaris*) contain an endo-β-1,3-glucanase as well as a chitinase (1). Chitin and β-1,3-glucans are polymers known to be in cell walls of fungi (7). Recently, Pegg and Vessey (18) have demonstrated that the chitinase present in tomatoes is able to degrade the cell wall and thereby cause lysis of the tomato fungal pathogen *Verticillium albo-atrum*. Abeles *et al.* (1) have demonstrated that the activity of chitinase and of endo-β-1-,3-glucanase in bean leaves increases dramatically (up to 50 times) following treatment of the leaves with ethylene. As these workers point out, an increase in ethylene production has been associated with viral (6, 16), bacterial (11, 15), and fungal infections of plant tissues (21). This effect of ethylene suggests that a defense mechanism of plants is their ability to synthesize enzymes capable of degrading the cell walls of their pathogens. We decided to determine whether *C. lindemuthianum* secretes inhibitors of the enzymes within the bean plant which would be capable of attacking the cell walls of this pathogen.

METHODS AND RESULTS

Since the composition and structure of the cell walls of *Colletotrichum lindemuthianum* had not previously been reported, we undertook to isolate cell walls from the mycelia of this fungus (22). The mycelia were ground in a Sorvall Omnimixer and then in a Waring Blendor in 5 volumes of 50 mM potassium phosphate, pH 7.0, at 2 C. The mycelial walls were suspended in 1% sodium dodecyl sulfate and stirred at 93 C for 0.5 hr. The cell wall pellet was washed repeatedly with buffer, then with a mixture of chloroform-methanol (1:1), and finally with acetone before being air dried at room temperature.

The sugar composition of the mycelial cell walls was determined by acid hydrolysis followed by the formation of the corresponding alditol acetates and separation of these derivatives by quantitative gas-liquid chromatography (5, 12). The results of this analysis (Fig. 1) demonstrate that the carbohydrate portion of the cell walls of *C. lindemuthianum* mycelia is largely glucose-containing polymers (91%). In addition to glucose, the walls contain small amounts of galactose (5%) and mannose (4%). The chromatogram presented in Figure 1 was obtained following hydrolysis of the cell walls for 1 hr at 121 C in 2 N trifluoroacetic acid. Hydrolysis of the glucose polymers under these conditions suggests that they are not cellulose-like. Hydrolysis of the mycelial cell walls for 2 hr at 121 C in 6 N trifluoroacetic acid results in a lower yield of glucose, mannose, and galactose. The absence of glucosamine in the hydrolyzed cell wall preparation demonstrates that the walls of *C. lindemuthianum* do not contain chitin-like polymers.

The glycosyl linkages in the cell wall polymers of *C. lindemuthianum* were determined following methylation analysis and the formation of partially methylated alditol acetates by procedures previously described (8, 20). The major partially methylated alditol acetate obtained in this procedure (Fig. 2) corresponds to the derivative of 3-linked glucosyl residues.

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Four-linked glucosyl residues are the second most prevalent wall constituent, about one of these residues being present for every five residues of 3-linked glucose. Identification of these derivatives was achieved by comparison of their retention times with the retention times of two internal standards (Fig. 2) and by mass spectrometric analysis of the gas chromatographic effluent (20). Thus, cell walls of *C. lindemuthianum* are largely composed of glucans rich in 1,3-glucosyl linkages but which also contain some 1,4-linked glucosyl residues. By analogy with previous studies of fungal cell walls, we assume that all of the glucosyl residues are in the form of the β anomer (7).

In order to determine whether *C. lindemuthianum* produces inhibitors of the β-1,3-endoglucanase and of the chitinase, we partially purified these enzymes following the procedures described by Abeles *et al.* (1) through the ammonium sulfate precipitation step. Our standard assay for the endoglucanase contains sufficient enzyme to yield an absorbance of 0.5 in the undiluted Nelson (17)-Somogyi (19) reducing group test after incubation of enzyme and substrate at 50 C for 1 hr. The substrate in the standard reaction mixture was 200 μl of 0.5% laminarin that had been reduced with sodium borohydride,

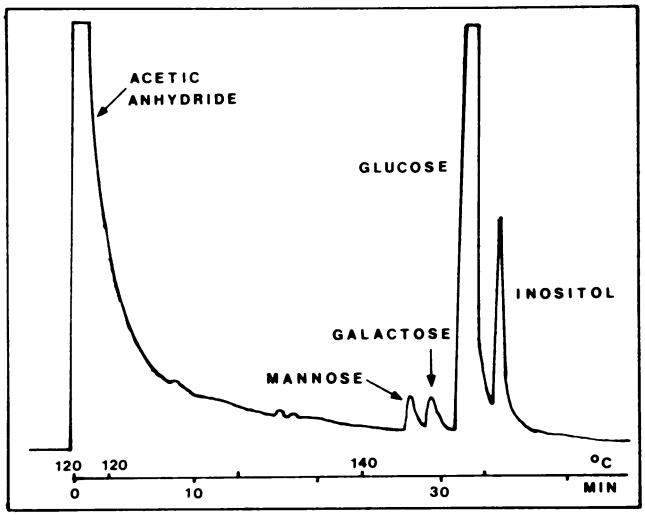

FIG. 1. Gas chromatogram of the alditol acetates derived from *C. lindemuthianum* mycelia cell walls following 2 N trifluoroacetic acid hydrolysis. Inositol is an internal standard. The analysis is by methods previously described (5, 12).

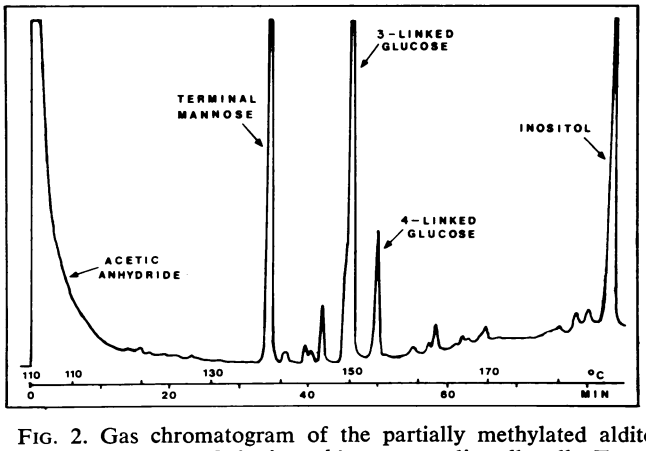

FIG. 2. Gas chromatogram of the partially methylated alditol acetates derived from *C. lindemuthianum* mycelia cell walls. Terminal mannose and myoinositol are internal standards. Analysis by methods previously described (20).

then exhaustively dialyzed against 1 M acetic acid, against distilled water, and finally freeze-dried. This treatment of the laminarin removed more than 80% of the reducing groups present in the untreated laminarin. The reaction mixtures contained varying amounts of inhibitor in 50 mM potassium acetate, pH 5.0, and were adjusted to 1 ml by the addition of this buffer. The reactions were terminated by the addition of 1 ml of the Nelson-Somogyi copper reagent.

C. lindemuthianum was grown in shake cultures as described previously (9) with 0.46% casein hydrolysate and 1.5% sucrose as the nutrients. After 8 days of culturing at 25 C, the mycelia were collected on a coarse sintered-glass funnel and washed with water. The mycelia were then ground in a Waring Blendor with 2 volumes of water. The resulting suspension was centrifuged at 10,000*g* for 30 min and the supernatant dialyzed against 50 mM potassium acetate, pH 5.0.

The macromolecules present in the extracellular culture filtrate were precipitated by the addition of 3 volumes of 95% ethanol. This suspension was allowed to stand for 18 hr at 2 C. The precipitate was pelleted by centrifugation and the supernatant solution discarded. The pellet was extracted with 5 ml of water for each liter of extracellular culture fluid. The solubilized material was dialyzed against 50 mM potassium acetate, pH 5.0.

An inhibitor of the bean leaf endo-β-1,3-glucanase was found in the extracts of both the mycelia and the alcohol precipitate of the extracellular culture filtrate. No inhibitor of the bean leaf chitinase was detectable in either extract. Although endo-β-1,3-glucanase inhibitor activity is detectable in the potassium acetate extract of the alcohol-precipitated extracellular media, the amount of inhibitor activity is increased approximately 10-fold by heating the preparation at 93 C for 10 min. The amount of endoglucanase inhibitor present in the extracellular culture filtrate is variable but, in several experiments, 1 ml of the heated culture filtrate had at least enough inhibitor to inactivate completely the endoglucanase present in our standard assay. Although endoglucanase inhibitor is present in extracts of the mycelia as well as in the extracellular medium, the results presented in the remainder of this paper were obtained with the inhibitor isolated from the extracellular culture fluid.

The inhibitor secreted by *C. lindemuthianum* inactivates the bean leaf endoglucanase rapidly. Addition of inhibitor at various times during the time course of an assay stops, without measurable delay, the reaction between the endoglucanase and laminarin (Fig. 3). Inhibitor that had been partially purified by both DEAE[3]-Sephadex and Bio-Gel P-20 chromatography (see below) was used to obtain the data presented in Figure 3.

The endoglucanase inhibitor present in the extracellular medium of *C. lindemuthianum* was partially purified and characterized by both ion exchange and gel filtration chromatography. The inhibitor absorbs to a DEAE-Sephadex column (2.2 × 22 cm) when the column has been equilibrated with 200 mM potassium acetate, pH 5.0. Two partially separated peaks of inhibitor activity are eluted from the column by a linear ionic gradient formed from 200 ml of 200 mM potassium acetate, pH 5.0, and 200 ml of 500 mM potassium acetate, pH 5.0 (Fig. 4). Protein was determined by the method of Lowry *et al.* (14). The presence of two inhibitors has been demonstrated more clearly by collecting smaller fractions on a second DEAE-Sephadex column (data not presented). These active fractions are able to inhibit, to an equal extent, the β-1,3-glucanase extracted from the leaves of Dark Red Kidney beans and the

[3] Abbreviation: DEAE: diethylaminoethyl.

β-1,3-glucanase extracted from the leaves of Bush Blue Lake beans. This indicates that the β-1,3-glucanases in the leaves of Dark Red Kidney and Bush Blue Lake beans are similar to each other.

The fact that there are at least two forms of the β-1,3-glucanase inhibitor is confirmed by chromatography through Bio-Gel P-20 (Fig. 5). Two partially separated peaks of inhibitor activity are obtained by gel filtration through a 2.2- × 80-cm column. The two partially separated fungal proteins inhibit, to equal extents, the β-1,3-glucanases extracted from both the leaves of Dark Red Kidney and Bush Blue Lake beans. The two inhibitor proteins have approximate mol wt of

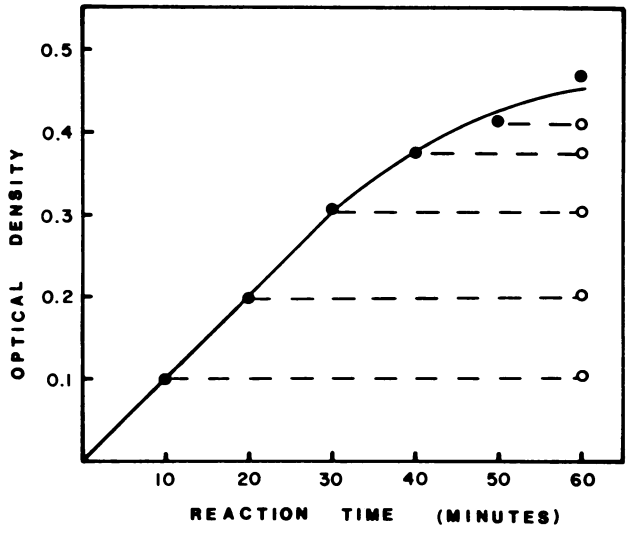

FIG. 3. Time course of endo-β-1,3-glucanase hydrolysis of laminarin. The absorbance is proportional to the glycosidic linkages hydrolyzed. See text for the details of analysis. Data obtained by stopping the reactions with the Nelson-Somogyi copper reagent (●); data obtained in duplicate reactions (○) to which *C. lindemuthianum* inhibitor was added at the time the copper reagent was added to the other reactions. Copper reagent was added to the reactions (○) after a total of 60 min.

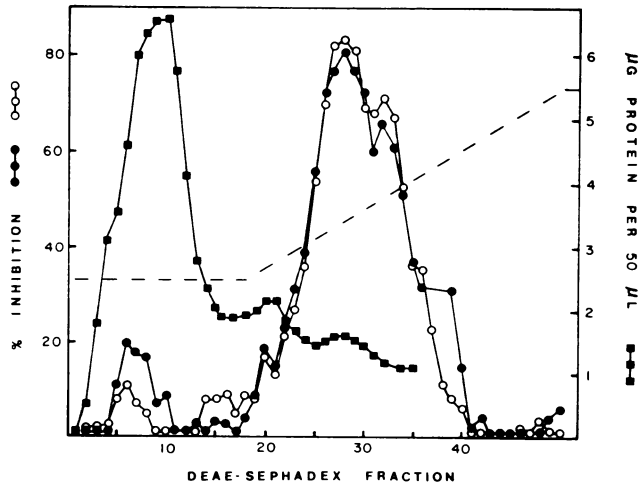

FIG. 4. DEAE-cellulose gradient chromatography of the *C. lindemuthianum* α strain proteins which inhibit the endo-β-1,3-glucanases of the leaves of Dark Red Kidney (○) and Bush Blue Lake (●) beans. The dashed line is proportional to the ionic strength which is increased from 200 mM to 500 mM potassium acetate, pH 5.0. Fractions were 10 ml.

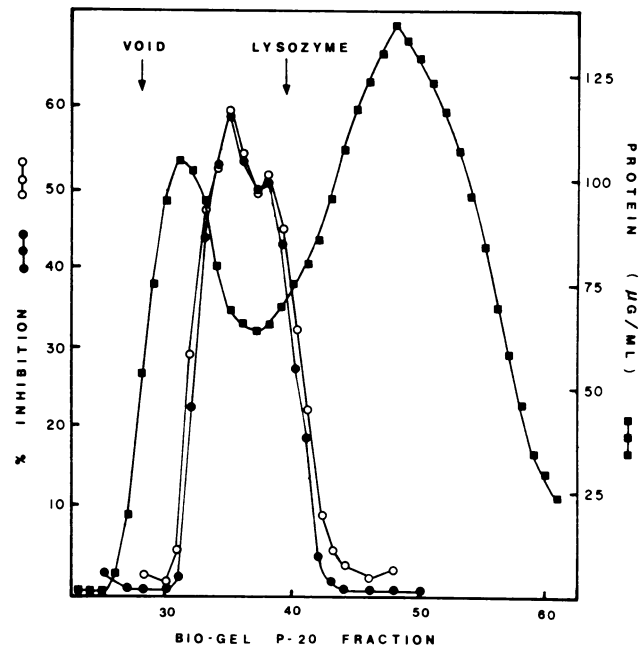

FIG. 5. Bio-Gel P-20 chromatography of the *C. lindemuthianum* α strain proteins which inhibit the endo-β-1,3-glucanases of the leaves of Dark Red Kidney (○) and Bush Blue Lake (●) beans. Fractions were 5 ml.

16,000 and 18,000 if one compares the retention time of these molecules on the Bio-Gel P-20 column with that of lysozyme and assumes that the inhibitor molecules are globular. As both forms of the β-glucanase inhibitor are inactivated by treatment with trypsin or pronase, we have concluded that the inhibitors are at least partially peptide in nature. This is further suggested by the fact that although the inhibitors are stable to heating at 93 C for 10 min, autoclaving at 121 C rapidly destroys their ability to inactivate the endoglucanase.

DISCUSSION

The results presented in this paper demonstrate that a fungal pathogen of plants has the ability to secrete protein-containing compounds which can effectively inhibit enzymes within plants that are potentially capable of attacking the pathogen. Preliminary results (not reported here) suggest that effective degradation of the cell walls of the pathogen requires an endo-β-1,4-glucanase as well as an endo-β-1,3-glucanase. More detailed studies of the plant's ability to degrade the cell walls of its pathogens are planned. The results that are reported demonstrate that not only have plants developed the ability to synthesize proteins capable of inhibiting degradative enzymes secreted by their pathogens, but the pathogens have apparently evolved a similar mechanism to assist them in invading plants. Whether or not these interacting systems can explain, at least in part, the specificity exhibited by many host-pathogen systems is a question worthy of detailed investigation.

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LITERATURE CITED

1. ABELES, F. B., R. P. BOSSHART, L. E. FORRENCE, AND W. H. HABIG. 1970. Preparation and purification of glucanase and chitinase from bean leaves. Plant Physiol. 47: 129-134.
2. ABELES, F. B. AND L. E. FORRENCE. 1970. Temporal and hormonal control of β-1,3-glucanase in *Phaseolus vulgaris* L. Plant Physiol. 45: 395-400.

3. ALBERSHEIM, P. AND A. J. ANDERSON. 1971. Host-pathogen interactions. III. Proteins from plant cell walls inhibit enzymes secreted by plant pathogens. Proc. Nat. Acad. Sci. U. S. A. 68: 1815–1819.
4. ALBERSHEIM, P., T. M. JONES, AND P. D. ENGLISH. 1969. Biochemistry of the cell wall in relation to infective processes. Annu. Rev. Phytopathol. 7: 171–194.
5. ALBERSHEIM, P., D. J. NEVINS, P. D. ENGLISH, AND A. KARR. 1967. A method for the analysis of sugars in plant cell-wall polysaccharides by gas-liquid chromatography. Carbohyd. Res. 5: 340–345.
6. BALAZS, E., R. GABORJANYI, A. TOTH, AND Z. KIRALY. 1969. Ethylene production in *Xanthi* tobacco after systemic and local virus infections. Acta Phytopathol. Acad. Sci. Hung. 4: 335–358.
7. BARTNICKI-GARCIA, S. 1968. Cell wall chemistry, morphogenesis, and taxonomy of fungi. Annu. Rev. Microbiol. 22: 87–108.
8. BJÖRNDAL, H., C. G. HELLERQUIST, B. LINDBERG, AND S. SVENSSON. 1970. Gas-liquid chromatography and mass spectrometry in methylation analysis of polysaccharides. Angew. Chem. Int. Ed. Engl. 9: 610–619.
9. ENGLISH, P. D., J. B. JURALE, AND P. ALBERSHEIM. 1971. Host-pathogen interactions. II. Parameters affecting polysaccharide-degrading enzyme secretion by *Colletotrichum lindemuthianum* grown in culture. Plant Physiol. 47: 1–6.
10. ENGLISH, P. D., A. MAGLOTHIN, K. KEEGSTRA, AND P. ALBERSHEIM. 1972. A cell wall-degrading endopolygalacturonase secreted by *Colletotrichum lindemuthianum*. Plant Physiol. 49: 293–297.
11. FREEBAIRN, H. T. AND I. W. BUDDENHAGEN. 1964. Ethylene production by *Pseudomonas solanacearum*. Nature 202: 313–314.
12. JONES, T. M. AND P. ALBERSHEIM. 1972. A gas chromatographic method for the determination of aldose and uronic acid constituents of plant cell wall polysaccharides. Plant Physiol. 49: 926–936.
13. JONES, T. M., A. J. ANDERSON, AND P. ALBERSHEIM. 1972. Host-pathogen interactions. IV. Studies on the polysaccharide-degrading enzymes secreted by *Fusarium oxysporum* f. sp. *lycopersici*. Physiol. Plant Pathol. 2: 153–166.
14. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193: 265–275.
15. LUND, B. M. AND L. W. MAPSON. 1970. Stimulation by *Erwinia carotovora* of the synthesis of ethylene in cauliflower tissue. Biochem. J. 119: 251–263.
16. NAKAGAKI, Y., T. HIRAI, AND M. A. STAHMANN. 1970. Ethylene production by detached leaves infected with tobacco mosaic virus. Virology 40: 1–9.
17. NELSON, N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153: 375–380.
18. PEGG, G. F. AND J. C. VESSEY. 1973. Chitinase activity in *Lycopersicon esculentum* and its relationship to the *in vivo* lysis of *Verticillium albo-atrum* mycelium. Physiol. Plant Pathol. 3: 207–222.
19. SOMOGYI, M. 1952. Notes on sugar determination. J. Biol. Chem. 195: 19–23.
20. TALMADGE, K. W., K. KEEGSTRA, W. D. BAUER, AND P. ALBERSHEIM. 1973. The structure of plant cell walls. I. The macromolecular components of the walls of suspension-cultured sycamore cells with a detailed analysis of the pectic polysaccarides. Plant Physiol. 51: 158–173.
21. WILLIAMSON, C. E. 1950. Ethylene, a metabolic product of diseased and injured plants. Phytopathology 40: 205–208.
22. ZEVENHUIZEN, L. P. T. M. AND S. BARTNICKI-GARCIA. 1969. Chemical structure of the insoluble hyphal wall glucan of *Phytophthora cinnamomi*. Biochemistry 8: 1496–1502.