MASTER

Botany

Host-Pathogen Interactions III.

Proteins from Plant Cell Walls Inhibit Enzymes
Secreted by Plant Pathogens

by

Peter Albersheim and Anne J. Anderson
Department of Chemistry, University of Colorado
Boulder, Colorado 80302

Running title: Host-Pathogen Interactions

This report was prepared as an account of work sponsored by the United States Government. Neither the United States nor the United States Atomic Energy Commission, nor any of their employees, nor any of their contractors, subcontractors, or their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness or usefulness of any information, apparatus, product or process disclosed, or represents that its use would not infringe privately owned rights.

leg

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency Thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

ABSTRACT

Proteins have been extracted from the cell walls of Red Kidney bean hypocotyls, tomato stems, and suspension-cultured sycamore cells which can inhibit completely the activity of the polygalacturonases secreted by the plant pathogens, Colletotrichum lindemuthianum, Fusarium oxysporum and Sclerotium rolfsii. The inhibitor of the C. lindemuthianum polygalacturonase has been purified 560-fold from bean hypocotyl extracts. This purified protein is 40 times more effective an inhibitor of the C. lindemuthianum polygalacturonase than of the F. oxysporum polygalacturonase, and does not demonstrably inhibit the S. rolfsii polygalacturonase. A crude hypocotyl extract capable of inhibiting completely the three polygalacturonases does not inhibit C. lindemuthianum-secreted cellulase, xylanase, α -galactosidase, α -arabinofuranosidase, or α -galacturonosidase. The purified bean hypocotyl protein combines with the C. lindemuthianum polygalacturonase to form a complex with a dissociation constant of 2×10^{-9} M or less. The physical properties of these inhibitors are very similar to those of phytohemagglutinins and of the plant glycoproteins capable of agglutinating transformed animal cells.

INTRODUCTION

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 we would propose 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 universally have the ability to 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 into their culture medium polysaccharide degrading enzymes. 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 secreted wall degrading enzymes into its culture medium in a sequential manner. The first enzyme to be secreted in this case is also a polygalacturonic acid degrading enzyme. The results described in the present paper demonstrate in the cell walls of Red Kidney bean hypocotyls, of tomato stems, and of sycamore cells grown in suspension culture the presence of proteins capable of inhibiting 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

Growth of plant material and culture of pathogens: 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°, 1.25 µmoles 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.

Cxysporum f. sp. lycopersici was cultured according to published methods (9), but the production of extracellular enzymes was carried out 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% de-esterified (pH 12, 0°, 2 hr) and sodium borohydride reduced (10 mg/liter, pH 12, 0°, 1 hr) citrus pectin. The activities of xylanase and of cellulase were measured in 0.1% solutions of xylan (Koch-Light Laboratories, Ltd.) and of carboxymethylcellulose (Hercules, Inc.), respectively. The α -pectinase was also measured viscometrically (7). The activities of highly purified C. lindemuthianum α -galactosidase 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 using sufficient inhibitor to reduce the rate of polygalacturonic acid degradation by 50%. One unit of inhibitor is defined as that amount which prevents the formation of 0.7 μ moles of galacturonic acid reducing group equivalents in 1 hr at 30° when the reaction is carried out in 50 mM sodium acetate, pH 5.2, and in a total volume of 1 ml. This solution usually contains 800 μ g of polygalacturonic acid (equivalent to 4.5 μ moles of galacturonic acid), and an amount of polygalacturonase which produces about 1.4 μ moles of

galacturonic acid reducing equivalents in the absence of inhibitor.

Proteins were measured by the method of Lowry et al. (11).

All experiments were carried out at 2° unless otherwise specified.

Standard buffers: The standard buffer used in these studies is 50 mM sodium acetate, pH 5.2. This solution is referred to in this manuscript as "Buffer". Buffer that contains 1 mM each of CaCl_2 , MnCl_2 and MgCl_2 is referred to as "Buffer plus Metals".

Extraction and purification of inhibitor from Red Kidney bean hypocotyls:

The hypocotyl protein which inhibits the C. lindemuthianum polygalacturonase was extracted as described below. Five hundred g of 8-day-old hypocotyls were harvested and chopped into 2 to 4 mm segments with a razor blade. The segments and 1 l of 0.5 M potassium phosphate, pH 7, were homogenized for 60 sec in a 1 gal Waring Blendor. The resulting suspension was placed in a 4 l coarse sintered glass funnel and the liquid portion allowed to pass through the funnel without suction. The residue was homogenized twice more, first with 750 ml and then with 500 ml of the phosphate buffer. The extracts were combined (2230 ml) and adjusted to pH 4.6 with 4 N HCl. After 30 min, the solution was readjusted to pH 7 with 4 NaOH and the insoluble material removed by centrifugation for 30 min at 13,000 x g.

The supernatant fraction was adjusted to 20% of saturation with $(\text{NH}_4)_2\text{SO}_4$ and stirred for 30 min. The resulting suspension was centrifuged at 13,000 x g for 20 min and the pellet discarded. The supernatant liquid was subjected to further $(\text{NH}_4)_2\text{SO}_4$ fractionation to yield those proteins insoluble

in 20-40% and 40-70% $(\text{NH}_4)_2\text{SO}_4$. 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 x g. The insoluble material was washed with 5 ml of 5 mM sodium acetate, pH 5.2, and the wash added to the supernatant fraction. This soluble portion was passed through a DEAE-cellulose column (1.3 x 12 cm) that had previously been equilibrated with the 5 mM buffer. The material which 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 x 14 cm). The Sephadex was not used for its ability to size molecules but rather 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 a 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 per ml in each of the 12 ml fractions collected from this gradient elution were assayed. The results are summarized in Fig. 1. Fractions 90 through 98 were combined and dialyzed against Buffer plus Metals. In order to concentrate the inhibitor, the combined fractions were passed through a second Sephadex G-25-300 column (1.6 x 6 cm). The solution which passed through this column was discarded. The

Fig. 2

column was then eluted with a solution containing Buffer plus Metals and 300 mM NaCl. One ml fractions were collected and assayed for inhibitor. Fractions 6 to 11 were combined and 4.8 ml of this solution was chromatographed on a Bio Gel P-100 column (2.2 x 89 cm, void volume of 96 ml) by eluting with a solution containing Buffer plus Metals and 100 mM NaCl. The protein and inhibitor concentrations per ml in each of the 12 ml fractions were assayed. Fraction 12, 13, and 14 were combined and the inhibitor concentrated on a Sephadex G-25-300 column (1.6 x 6 cm) as described above. This concentrate (5.0 ml) was applied to a Bio Gel P-150 column (1.4 x 44 cm, void volume of 26 ml). The column was eluted with a solution containing Buffer plus Metals and 100 mM NaCl. The protein and inhibitor concentrations per ml in each of the 6.2 ml fractions were assayed. The results are summarized in Fig. 2.

Extraction of polygalacturonase inhibitors from sycamore cells: Suspension cultured sycamore (Acer pseudoplatanus) cells were collected on a coarse sintered glass funnel and washed three times with 10 volumes of 10 mM potassium phosphate, pH 7. The inhibitor was then extracted by suspending the washed cells in 3 volumes of 500 mM potassium phosphate, pH 7. The soluble portion was sucked through the filter by reduced pressure and the cells once again suspended in 3 volumes of the 500 mM phosphate buffer. This second extract was added to the first and the solution adjusted to 70% of saturation with respect to $(\text{NH}_4)_2\text{SO}_4$. The resulting precipitate was collected by centrifugation at 46,000 x g for 20 min. The pellet was suspended in Buffer; the soluble portion contained the polygalacturonase inhibitors.

Extraction of polygalacturonase inhibitors from tomato stems: Six-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 Blendor for 60 sec. The supernatant solution was removed by filtration through a coarse sintered glass funnel and discarded. The residue was homogenized once again with 3 volumes of the 100 mM phosphate buffer, and again the supernatant fraction was discarded. The residue was ground with 3 volumes of 500 mM potassium phosphate, pH 7, and the supernatant fraction collected by filtration. The residue was extracted again with 3 volumes of the 500 mM phosphate buffer and the supernatant fraction combined with the first 500 mM buffer extract. This solution was adjusted to 70% with respect to $(\text{NH}_4)_2\text{SO}_4$. The resulting precipitate was collected by centrifugation for 5 min at 48,000 x g. The pellet was suspended in Buffer; the soluble portion contained the polygalacturonase inhibitors.

RESULTS

Purification of the Red Kidney bean hypocotyl protein which inhibits the polygalacturonase secreted by C. lindemuthianum is summarized in Table 1. 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.

Preincubation of the inhibitor with the polygalacturonase is not required for effective inhibition: This is demonstrated by the results of an experiment which compared the ability of the purified Red Kidney hypocotyl protein with the ability of the Nelson-Somogyi copper reagent to terminate the C. lindemuthianum polygalacturonase catalyzed hydrolysis of polygalacturonic

acid. The results of this experiment indicate that addition of the inhibitor stops the enzymic reaction at least as quickly as addition of the alkaline copper reagent.

Fig. 3
The degree of polygalacturonase inhibition is dependent on the amount of inhibitor added: This is illustrated by the data summarized in Fig. 3. As expected, the reaction rate is dependent on the substrate concentration. Polygalacturonic acid concentrations above 0.06% result in substrate inhibition (Fig. 4). Nevertheless, the substrate concentration has little effect on the degree of inhibition resulting from the presence of the Red Kidney bean inhibitor (Fig. 4).

Fig. 5
Red Kidney bean hypocotyl inhibitor is relatively stable to heat: Heating a solution of the polygalacturonase inhibitor for 210 min at 50° destroys only about 16% of its activity (Fig. 5). About 77% of the inhibitor activity is lost by heating for 210 min at 55°. Essentially all of the activity is destroyed after 15 min at 70°.

Extracts of the cell walls of tomato stems, sycamore cells and bean hypocotyls are each capable of inhibiting the polygalacturonases secreted by *C. lindemuthianum*, *F. oxysporum* and *S. rolfsii*: The 0-70% $(\text{NH}_4)_2\text{SO}_4$ precipitates of the extracts from the cell walls of "Clark's Special" and "Jefferson" tomato stems, of sycamore cells and of Red Kidney bean hypocotyls have the ability to inhibit completely the activity of the polygalacturonases secreted by *C. lindemuthianum*, *F. oxysporum* and *S. rolfsii*. The extracts

Table 2
possess different abilities to inhibit the three polygalacturonases (Table 2). 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 multiple inhibitors within a given plant.

The presence of multiple inhibitors with different abilities to inhibit each of the three fungal polygalacturonases is confirmed by the relative inability of the C. lindemuthianum polygalacturonase inhibitor purified from Red Kidney bean hypocotyls to inhibit the F. oxysporum and S. rolfsii polygalacturonases (Table 2). While the inhibitors in the relatively impure 0-70% $(\text{NH}_4)_2\text{SO}_4$ precipitate of the Red Kidney hypocotyl extract are most effective against the C. lindemuthianum enzyme, inhibition of this enzyme is only ten times more effective than inhibition of the F. oxysporum enzyme and only four times more effective than inhibition of the S. rolfsii polygalacturonase. However, the purified hypocotyl protein is forty times more effective an inhibitor of the C. lindemuthianum polygalacturonase than of the F. oxysporum polygalacturonase and it has no measurable ability to retard the action of the S. rolfsii polygalacturonase.

Further evidence of the multiplicity of inhibitors comes from the observation that the Red Kidney hypocotyl proteins which inhibit the C. lindemuthianum and F. oxysporum polygalacturonases can be physically separated from the protein which inhibits the S. rolfsii polygalacturonases. The inhibitors of the C. lindemuthianum and F. oxysporum enzymes are extracted from hypocotyl cell walls by 50 mM sodium acetate, pH 5.2, containing 300

mM NaCl. The S. rolfsii polygalacturonase inhibitor, under these conditions, remains bound to the hypocotyl walls, but this inhibitor is extracted in 500 mM potassium phosphate, pH 7.

The Red Kidney bean hypocotyl extracts do not inhibit other polysaccharide degrading enzymes: The 0-70% $(\text{NH}_4)_2\text{SO}_4$ precipitate of the Red Kidney bean hypocotyl extracts was used in an attempt to inhibit purified preparations of α -arabinosidase, α -galactosidase, carboxymethylcellulase, xylanase and exo-polygalacturonosidase. None of these five enzymes, which are secreted by C. lindemuthianum during growth on isolated cell walls, is inhibited detectably by the hypocotyl extracts, although the same extracts can completely inhibit the endopolygalacturonases.

DISCUSSION

The C. lindemuthianum polygalacturonase inhibitor isolated from Red Kidney bean hypocotyls has a number of properties which suggests that it is one of the glycoproteins commonly referred to as phytoagglutinins. The heat stability of the inhibitor is comparable to that of the phytoagglutinins (e.g., 5, 12). The approximate molecular weight of this protein (50,000 from its elution characteristics on Bio Gel P-100 and P-150) is in agreement with those for a number of phytoagglutinins (12, 13, 14). In common with the phytoagglutinins, the polygalacturonase inhibitor fails to bind to DEAE-cellulose but binds to carboxymethyl cation exchange columns (12, 14, 15, 16, 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 red blood cells has been shown repeatedly (15, 17, 20, 21, 22). Tunis (23) has demonstrated that the seeds contain a protein which agglutinates transformed animal cells but not red blood cells. Thus, Red Kidney beans have agglutinins, including perhaps the polygalacturonase inhibitors, which do not agglutinate red blood cells.

The suggestion that the polygalacturonase inhibitors are phytoagglutinins would be more firmly established if a hapten for the inhibition reaction could be identified. A large number of carbohydrates were tested for their ability to prevent the protein from Red Kidney bean hypocotyls from inhibiting the C. lindemuthianum polygalacturonase. The failure to find such an antagonist may be a consequence of the inhibitor binding to a complex oligosaccharide on the surface of the enzyme rather than to a simple sugar (24). A hapten, which 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 ml reaction mixture, less than 10^{-11} moles of the inhibitor completely inactivates about 3×10^{-13} moles of the polygalacturonase; from such data we have calculated a maximum dissociation constant of 2×10^{-9} 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 which inhibit other degradative enzymes with a higher degree of specificity. These include the soybean trypsin inhibitors (28, 29) and the invertase inhibitors from potatoes and beets (30).

The results of this paper demonstrate that plant cell walls contain a variety of proteins which inhibit pathogen secreted endopolygalacturonases without inhibiting any of a number of other enzymes tested. And these inhibitors distinguish between polygalacturonases secreted by different species of pathogenic fungi. This suggests that such inhibitors participate in disease resistance.

Acknowledgements

The authors are indebted to Wolfgang D. Bauer, Max Burger, and Kenneth Keegstra for stimulating discussions which led to the experiments described in this paper.

This work has been supported by Atomic Energy Commission Contract #AT(11-1)-1426 and by a University of Colorado Faculty Fellowship.

REFERENCES

1. Mäkelä, O., Ann. Med. Exp. et Biol. Fenn., 35, Supp. 1-12, (1957).
2. Boyd, W.C., Vox Sang., 8, 1 (1963).
3. Liener, I.E., Econ. Botan., 18, 27 (1964).
4. Aub, J.C., C. Tieslau, and A. Lankester, Proc. Nat. Acad. Sci. USA, 50, 613 (1963).
5. Burger, M.M., and A.R. Goldberg, Proc. Nat. Acad. Sci., USA, 57, 359 (1967).
6. Albersheim, P., T.M. Jones, and P.D. English, Ann. Rev. Phytopathol., 7, 171 (1969).
7. English, P.D., B. Jurale, and P. Albersheim, Plant Physiol., 47, 1 (1971).
8. Bateman, D.F., Neth. J. Pl. Path., 74, (1968 Supp.), 67 (1968).
9. Patil, S.S., and A.E. Dimond, Phytopathology, 58, 676 (1968).
10. Somogyi, M., J. Biol. Chem., 195, 19 (1952).
11. Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall, J. Biol. Chem., 193, 265 (1951).
12. Howard, I.K., and H.J. Sage, Biochemistry, 8, 2436 (1969).
13. Ticha, M., G. Entlicher, J.V. Kostir, and J. Kocourek, Biochim. Biophys. Acta, 221, 282 (1970).
14. Entlicher, G., J.V. Kostir, and J. Kocourek, Biochim. Biophys. Acta, 211, 272 (1970).
15. Takahashi, T., P. Ramachandramurthy, and I.E. Liener, Biochim. Biophys. Acta, 133, 123 (1967).
16. Howard, I.K., H.J. Sage, M.D. Stein, N.M. Young, M.A. Leon, and D.F. Dyckes, J. Biol. Chem., 246, 1590 (1971).
17. Dahlgren, K., J. Porath, and K. Lindahl-Kiessling, Arch. Biochem. Biophys., 137, 306 (1970).

18. Agrawal, B.B.L., and I.J. Goldstein, Biochim. Biophys. Acta, 147, 262 (1967).
19. Olson, M.O.J., and I.E. Liener, Biochemistry, 6, 105 (1967).
20. Jaffe, W.G., and K. Hannig, Arch. Biochem. Biophys., 109, 80 (1965).
21. Li, J.G., and E.E. Osgood, Blood, 4, 670 (1949).
22. Rigas, D.A., and E.E. Osgood, J. Biol. Chem., 212, 607 (1955).
23. Tunis, M., J. Immunol., 92, 864 (1964).
24. Kornfeld, R., and S. Kornfeld, J. Biol. Chem., 245, 2536 (1970).
25. Bell, T.A., J.L. Etchells, and W.W.G. Smart, Jr., Botan. Gaz., 126, 40 (1965).
26. Cook, C.E., J.A. Buhrman, C.R. Tallent, and M.E. Wall, Lloydia, 33, 255 (1970).
27. Weurman, C., Acta Botanica Neerlandica, 2, 107 (1953).
28. Kunitz, M., J. Gen. Physl., 30, 291 (1947).
29. Frattali, V., and R.F. Steiner, Biochemistry, 7, 521 (1968).
30. Pressey, R., Plant Physiol. 43, 1430 (1968).

LEGENDS FOR FIGURES

Figure 1. Sephadex G-25-300 chromatography of the Red Kidney hypocotyl protein which inhibits the C. lindemuthianum polygalacturonase. The shaded fractions are those combined for Bio Gel P-100 chromatography. See Materials and Methods for details.

Figure 2. Bio Gel P-150 chromatography of the Red Kidney bean hypocotyl protein which inhibits the C. lindemuthianum polygalacturonase. The shaded fractions are those combined and used for some of the experiments described in Results. See Materials and Methods for details.

Figure 3. The percent inhibition of C. lindemuthianum polygalacturonase (0.1 μ g in 50 μ l) by varying amounts (in 50 μ l) of purified Red Kidney hypocotyl protein. The reactions were incubated at 30° for 1 hr and contained 900 μ g of polygalacturonic acid in 0.9 ml of Buffer. See Materials and Methods for details of the assay.

Figure 4. The effect of substrate (polygalacturonic acid) concentration (in 0.9 ml) on the rate of its hydrolysis by C. lindemuthianum polygalacturonase (0.1 μ g in 50 μ l). The effect of adding 50 μ l of the purified Red Kidney hypocotyl protein which inhibits this reaction is also presented. The several inhibitor concentrations in μ g per ml are indicated as: (●), no inhibitor; (○), 0.53; (■), 0.74; and (□), 1.05. See Materials and Methods for details of the assay.

Figure 5. The effect of preheating the 0-70% $(\text{NH}_4)_2\text{SO}_4$ fraction of the Red Kidney hypocotyl protein on its ability to inhibit the hydrolysis of polygalacturonic acid by the C. lindemuthianum polygalacturonase. The reaction mixtures contained 100 μg of inhibitor protein in 50 μl , 0.1 μg of enzyme in 50 μl , and 900 μg polygalacturonase acid in 0.9 ml of Buffer.

Table 1

Purification of the Protein from Red Kidney

Bean Hypocotyls which Inhibits the

C. lindemuthianum Polygalacturonase

Fraction	volume mls	total units*	yield	μg protein /ml	μg protein per unit of inhibitor	purification
Crude (dialyzed)	2230	27,000	100%	5,400	45	1.0
pH 4.6 supernatant	2500	32,000	120%	3,600	28	1.6
40-70% (NH ₄) ₂ SO ₄	80	17,350	64%	6,000	28	1.6
5 mM acetate dialysis	76	16,900	63%	2,400	11	4.1
DEAE-cellulose eluant	200	15,600	58%	470	6	7.5
Sephadex-gradient	85	7,000	26%	31	0.37	120
Sephadex concentrate	5.5	5,500	20%	350	0.35	130
Bio Gel P-100	48	3,694	14%	9	0.120	370
Bio Gel P-150	20	2,000	7%	8	0.08	560

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

Table 2
Amount of Protein Required for 50% Inhibition
of Three Polygalacturonases^a

Polygalacturonase Source	Inhibitor Source				
	Clark's Special Tomato Stems	Jefferson Tomato Stems	Sycamore Cells	Red Kidney Bean Hypocotyls (NH ₄) ₂ SO ₄ ppt.	Red Kidney Bean Hypocotyls "Purified"
<i>C. lindemuthianum</i>	1 ^b	1 ^b	4	1 ^d	1 ^e
<i>F. oxysporum</i>	9	5	9	10	40
<i>S. rolf sii</i>	6	5	1 ^c	4	>100 ^f

^a See Materials and Methods for assay conditions.

^b 14 µg of protein

^c 7 µg of protein

^d 28 µg of protein

^e 0.1 µg of protein

^f no measurable inhibitor activity with 10 µg of protein

Fig. 1

Fig. 2