Polygalacturonase Inhibition in Rind of Valencia Orange Infected with *Diplodia natalensis*

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ABSTRACT

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A protein that inhibited polygalacturonase (PG) secreted by *Diplodia natalensis* was isolated from extracts of noninfected and infected cultivar Valencia orange rind. The inhibitor had a molecular weight (determined by gel filtration) near 54,000 daltons and was inactivated by heat and protease. In vivo and in vitro inhibition of PG involved the formation under acidic conditions of an inhibitor-PG (INH-PG) complex with a molecular weight of about 95,000. The INH-PG complex was dissociated at pH 8.0 which restored PG activity when assayed for optimum activity at pH 5.0. Results of reaction kinetics studies of the inhibitor indicated that the mode of PG inhibition was competitive.

Additional key words: *Citrus sinensis*, pectolytic enzymes, postharvest decay.

The postharvest decay caused in oranges (*Citrus sinensis* (L.) Osbeck) by *Diplodia natalensis* Pole-Evans is associated with extracellular endopolygalacturonase (PG) (5). In a study of PG activity in lesions caused by *D. natalensis*, minimal PG activity was detected in the exudate even though tissue degradation was extensive. However, detection of the enzyme was increased substantially by adjusting the pH of the exudate from its initial level of pH 3.6 to pH 8.5 prior to assaying for optimum PG activity at pH 5.0 (5). Low recovery of pectolytic enzymes from fruit tissue infected by fungal pathogens is not uncommon, and has been attributed to the presence of inhibitors of these enzymes (8). Inhibitors of PG have also been isolated from noninfected plant tissue (1–3,9,10). Their role in disease resistance has not been clearly established.

This paper presents evidence of an inhibitor of PG in cultivar Valencia orange rind and defines the interaction between the inhibitor and PG in tissue decayed by *D. natalensis*.

MATERIALS AND METHODS

Cultivar Valencia oranges, surface sterilized in a solution of 1% sodium hypochlorite, were inoculated by inserting mycelia of *D. natalensis* into the mesocarp through an incision, and incubated for 5 days at 30 C. The decayed rind was removed and frozen until analyses were made.

Acetone powders were prepared from nondecayed and decayed tissue (80 g) as previously described (5). The powder was extracted with 0.1 M tris hydroxymethylaminomethane plus glycine (tris-glycine), pH 8.5, containing 2% NaCl by low-speed stirring with a Sorvall Omni-Mixer. The homogenate was centrifuged at 22,000 *g* for 15 min at 1 C and the pellet was discarded. Protein in the supernatant was precipitated with cold acetone (−10 C), collected by centrifugation, and dissolved in 0.05 M tris-glycine, pH 8.5, plus 2% NaCl. The extract (4.5 ml) was clarified by centrifugation, loaded on a Sephadex G-100 column (3 × 81 cm), and eluted with the latter buffer. An additional extract was prepared from acetone powder according to the same procedure except that the protein precipitate was dissolved in 0.50 M sodium citrate buffer, pH 3.6, containing 2% NaCl and chromatographed by gel filtration with the same system. The Sephadex column was eluted at 1.0 ml/min, and 5.1-ml fractions were collected. The extraction and chromatography procedures were conducted at or below 4 C. Protein content was determined by using the Bio-Rad Laboratories procedure (6).

Molecular weights of the inhibitor and PG were determined by gel filtration with Sephadex G-100 by the method of Andrews (4). Nondenatured protein molecular weight markers from 29,000 to 132,000 daltons were obtained from Sigma Chemical Company (St. Louis, MO). The elution profile of the molecular markers was determined for both elution buffers.

PG activity was assayed by the liberation of reducing groups from polygalacturonic acid (PGA), sodium salt, dissolved in sodium citrate buffer at pH 5.0. The buffering capacity of the PGA solution was adjusted as required to minimize a change in pH caused by the addition of various fractions to be assayed. The glucose equivalent of reducing groups was determined by the procedure of Nelson (12). Reaction mixtures were incubated at 37 C.

The inhibitor was assayed by measuring the reduction in PG activity. The PG used was obtained from decayed tissue fractionated on Sephadex G-100 at pH 8.5. A standard amount of PG was mixed with an aliquot of the various fractions, and the solution was adjusted to pH 3.6 by the addition of citric acid. PGA, buffered to pH 5.0, was then added to the solution and assayed for PG activity. The inhibitor-PG (INH-PG) complex isolated by gel filtration at pH 3.6 was assayed by adjusting the various fractions to pH 8.5 with NaOH, adding an aliquot to PGA, and assaying for PG activity at pH 5.0.

The relationship between pH and the dissociation of the INH-PG complex was studied by adjusting the pH of aliquots of the complex from pH 3.6 to pH 5.0, 6.0, 7.0, 8.0, or 8.5 with NaOH solution prior to adding PGA and assaying for PG activity at pH 5.0.

The effect of heat on the stability of the inhibitor was determined by incubating the inhibitor at 60 C and removing aliquots at 2-min intervals. Inhibitor was also incubated with protease (Sigma P 5380), 3 mg/ml, in 0.1 M tris-HCl buffer, pH 7.5, at 37 C for 18 hr. Inhibitor activity in the various fractions was assayed as described above. The relationship between inhibitor concentration and degree of inhibition of PG was studied by incubating various amounts of inhibitor with a fixed amount of PG. Mode of inhibition was determined by double reciprocal Lineweaver-Burke plot (7). Polygalacturonase from *Aspergillus niger* (Sigma P 5146) was also tested for inhibition by the inhibitor.

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RESULTS

An inhibitor of PG, secreted by *D. natalensis*, was extracted from noninfected and infected orange rind and partially purified by gel filtration on Sephadex G-100. Detection of the inhibitor was accomplished by combining PG from decayed tissue with the inhibitor at pH 3.6 prior to adding the mixture to PGA and assaying for PG activity at pH 5.0. As the pH of the inhibitor-PG mixture was increased from pH 3.6 to pH 8.0 prior to adding the substrate, the activity of the inhibitor decreased (Fig. 1). The molecular weight of the inhibitor from decayed tissue, as determined by gel filtration, varied with the pH of the elution buffer. At pH 8.5, the molecular weight was about 50,000 (Fig. 2A), and at pH 3.6 it was about 58,000 daltons (Fig. 2B). The molecular weight of the inhibitor from noninfected tissue was 54,000 at both pH 8.5 and 3.6. Degree of inhibition was dependent upon the amount of inhibitor added to a fixed amount of PG (Fig. 3). Lineweaver-Burke reciprocal plots of PG in the presence of inhibitor at various substrate concentrations at pH 5.0 indicated the inhibition of PG was competitive (Fig. 4).

Heating of the eluant containing the inhibitor for 6 min at 60 C resulted in about 58% inactivation of the inhibitor. The inhibitor

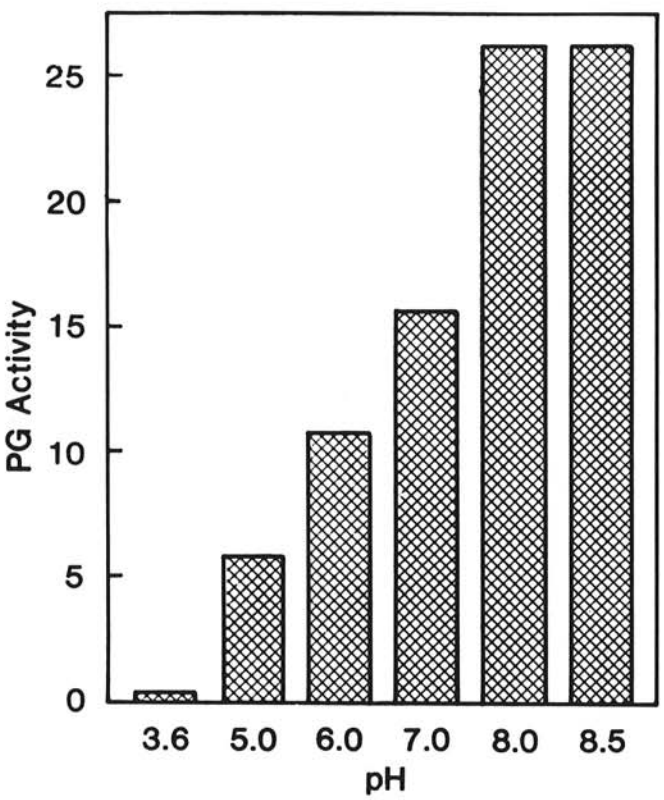

Fig. 1. Effect of pH on the dissociation of the inhibitor-PG complex in cultivar Valencia orange rind decayed by *Diplodia natalensis*. Polygalacturonase activity is expressed as micrograms of reducing groups liberated per 30 μl of eluant in 30 min. The pH of the inhibitor-PG fraction was adjusted before adding polygalacturonic acid and assaying for PG activity at pH 5.0.

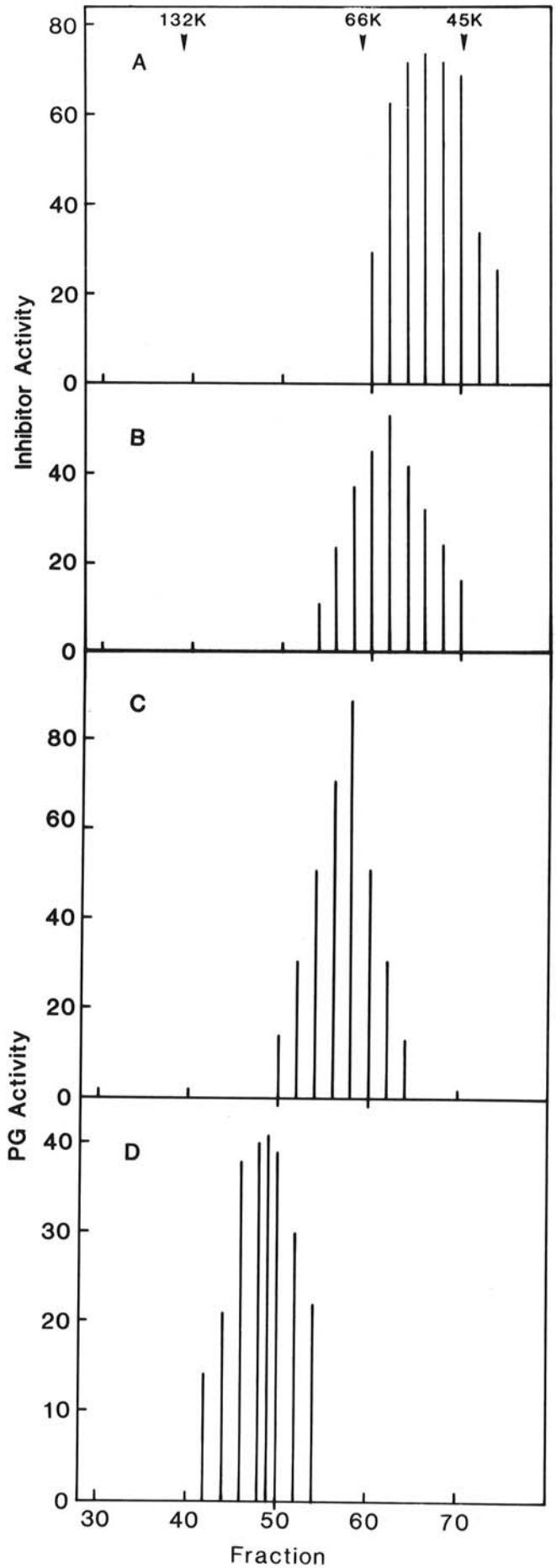

Fig. 2. Gel-filtration chromatography on Sephadex G-100 of crude protein extracts from cultivar Valencia orange rind decayed by *Diplodia natalensis*. **A and B:** Elution point of the inhibitor at pH 8.5 and 3.6, respectively. Inhibitor activity is expressed as the percentage reduction in a standard amount of PG activity. **C and D:** Elution point of PG and inhibitor-PG complex at pH 8.5 and 3.6, respectively. PG activity is expressed as micrograms of reducing groups liberated per 30 μl of eluant in 30 min. Elution buffers were 0.05 M tris-glycine, pH 8.5, and 0.50 M sodium citrate, pH 3.6, each containing 2% NaCl. Each fraction is equal to 5.1 ml. The elution points of the molecular weight markers are indicated by arrows at the top edge of **A**.

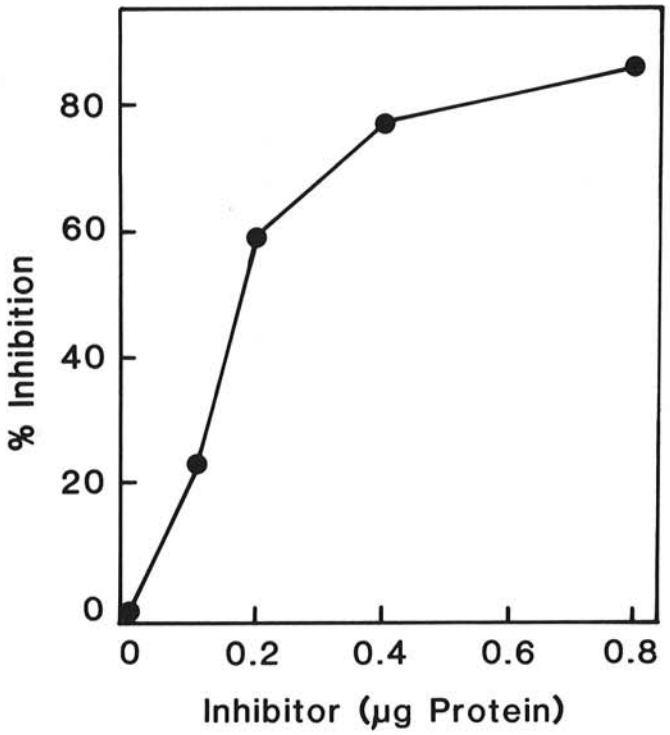

Fig. 3. Percentage inhibition of polygalacturonase (PG) produced by *Diplodia natalensis* by an inhibitor from orange rind decayed by the fungus. Protein concentration of the aliquot containing PG was 3.0 μg. Reaction time was 30 min.

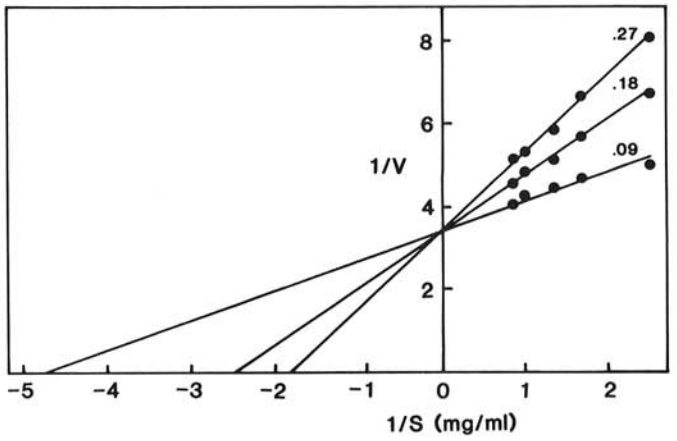

Fig. 4. Lineweaver-Burke double reciprocal transformation of polygalacturonase (PG) activity (micrograms of reducing groups liberated per 1.4 μg of protein in 20 min) in the presence of PG inhibitor (0.09, 0.18, 0.27 μg protein) at various concentrations of polygalacturonic acid.

was completely inactivated at 100 C. In addition, incubation of the inhibitor with protease for 18 hr resulted in 70.5% inactivation.

Extracts of decayed but not healthy tissue also contained PG activity. Molecular weight of the PG chromatographed by gel filtration at pH 8.5 was 65,000 (Fig. 2C). In contrast, when a similar extract was chromatographed at pH 3.6, no PG activity was detected in these fractions unless the fractions were adjusted to pH 8.5 prior to being assayed for PG activity. The molecular weight of the fraction containing PG activity was about 95,000 (Fig. 2D). This same extract also contained the inhibitor fraction shown in Fig. 2B. The same INH-PG complex was formed when the individual inhibitor and PG fractions, obtained by gel filtration at pH 8.5, were mixed and chromatographed at pH 3.6.

DISCUSSION

An inhibitor of PG was isolated from cultivar Valencia orange rind. The inhibitor is considered to be a protein on the basis of its molecular weight near 54,000 and its inactivation by heat and protease. Inhibitors of PG isolated from other plant tissues have also been identified as proteins, specifically glycoproteins (1–3). Evidence to suggest that the inhibitor present in cultivar Valencia rind is a glycoprotein was not obtained. The inhibitor efficiently inhibits PG secreted by both *D. natalensis* and *A. niger*. Other PG inhibitors have shown a broad specificity for PG (1,3,8–10).

The minimal level of PG activity detected in both exudate and extracts of lesions caused by *D. natalensis* is attributed to its inhibition by the inhibitor. The process of PG inhibition, in vitro and in vivo, involves the formation of an INH-PG complex under acidic conditions. Decayed tissue has a pH near 3.6 (5). Formation of this complex under acidic conditions and its dissociation at pH 8.0 would explain the observed effect of pH on the "activation" of PG in the exudate from lesions caused on cultivar Valencia oranges by *D. natalensis* (5).

The molecular weight of the INH-PG complex is about 95,000 which is less than the additive molecular weight, 123,000, of the PG (65,000) and inhibitor (58,000) in decayed tissue. The difference may be explained either by a change in the protein configuration upon formation of the complex, or by assuming that the inhibitor is composed of two subunits of approximately 29,000 each, one of which complexes with PG. An inhibitor of PG isolated from pear tissue has been reported to consist of two subunits of about 44,000 daltons each (1). Apparently, there is a sufficient quantity of inhibitor to complex and inactivate most all of the PG present in decayed tissue. Following gel filtration of a crude extract at pH 3.6, no PG activity was detected in any fraction with a molecular weight less than that of the INH-PG complex. However, an inhibitor fraction not complexed with PG was detected in the same extract.

The inhibitor in healthy tissue apparently has little or no effect on infection of the fruit or lesion development caused by *D. natalensis*. This decay develops very rapidly, and pectin degradation and tissue maceration are very extensive. Two factors may limit the role of the inhibitor in lesion development. First, an acidic condition is necessary for the inhibitor to complex with and inactivate PG. It is assumed that the pH of healthy, intercellular tissue is near neutral. Secondly, the inhibitor has a competitive mode of action, and a high substrate concentration at the lesion front would also limit the formation of the INH-PG complex. Thus, inhibition of PG at the site of infection or lesion front would be minimal, but the degree of inhibition as the lesion develops would increase as the pH decreases and the substrate becomes depleted. As previously suggested, inhibition of PG in the exudate formed within the lesion may be important in preventing the spread of this decay to a contacting healthy fruit (5). PG enzymes have been observed to damage cells of the epicarp of orange rind (*unpublished*) and injured tissue can serve as sites for infection by *D. natalensis* (11).

LITERATURE CITED

1. Abu-Goukh, A. A., Greve, L. C., and Labavitch, J. M. 1983. Purification and partial characterization of "Bartlett" pear fruit polygalacturonase inhibitor. Physiol. Plant Pathol. 23:111-122.
2. Albersheim, P., and Anderson, A. J. 1971. Proteins from plant cell walls inhibit polygalacturonase secreted by plant pathogens. Proc. Nat. Acad. Sci., USA 68:1815-1819.
3. Anderson, A. J., and Albersheim, P. 1972. Host-pathogen interactions. V. Comparison of the abilities of proteins isolated from three varieties of *Phaseolus vulgaris* to inhibit the endopolygalacturonases secreted by three races of *Colletotrichum lindemuthianum*. Physiol. Plant Pathol. 2:339-346.
4. Andrews, P. 1964. Estimation of molecular weights of proteins by Sephadex gel filtration. Biochem. J. 91:222-233.
5. Barmore, C. R., Snowden, S. E., and Brown, G. E. 1984. Endopolygalacturonase from Valencia oranges infected with *Diplodia natalensis*. Phytopathology 74:735-737.
6. Bio-Rad Laboratories. 1977. Bio-Rad protein assay. Tech. Bull. 1051. Richmond, CA. 4 pp.
7. Christensen, H. N., and Palmer, G. A. 1974. Enzyme kinetics. Second edition. W. B. Saunders Co., Philadelphia, PA.

8. Fielding, A. H. 1981. Natural inhibitors of fungal polygalacturonases in infected fruit tissue. J. Gen. Microbiol. 123:377-381.
9. Fisher, M. L., Anderson, A. J., and Albersheim, P. 1973. A single plant protein efficiently inhibits endopolygalacturonase secreted by *Colletotrichum lindemuthianum* and *Aspergillus niger*. Plant Physiol. 51:489-491.
10. Hoffman, R. M., and Turner, J. G. 1982. Partial purification of proteins from pea leaflets that inhibit *Ascochyta pisi* endopolygalacturonase. Physiol. Plant Pathol. 20:173-187.
11. Littauer, F., Nadel-Schiffmann, M., and Minz, G. 1947. The mode of infection of Shamouti oranges by *Diplodia natalensis*. Palestine J. Bot., Rehovot Ser. 6:158-164.
12. Nelson, N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 123:375-380.