

Endopolygalacturonase from Valencia Oranges Infected with *Diplodia natalensis*

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ABSTRACT

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The decay of oranges (*Citrus sinensis* 'Valencia') by *Diplodia natalensis* was found to be associated with two endopolygalacturonase (endo-PG) isozymes with molecular weights of 64,000 and 45,000 daltons. The endo-PG isozymes were apparently of fungal origin, since endo-PG was not detected in injured-uninfected rind, but was produced by the fungus during growth in vitro. Pectin degradation was aided by endogenous pectinmethylesterase (PE) which caused demethylation of pectin in the host cell walls at the edge of the lesion. In vitro production of PE by *D. natalensis* was not observed. Endo-PG activity in rind decayed by *D. natalensis* was

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substantially less than in comparable tissue infected with *Penicillium italicum*. In vitro production of endo-PG by *D. natalensis*, but not by *P. italicum*, was repressed during growth of the fungus. In addition, endo-PG activity in the exudate from tissue decayed by *D. natalensis* was minimal. However, endo-PG activity was substantially increased by adjusting the pH of the exudate from its initial level of pH 3.6 to pH 8.5 prior to assay at pH 5.0, optimum pH for enzyme activity. Cell walls at the edge of the lesion were slightly swollen, and maceration of tissue at the site of hyphal penetration was more extensive in the mesocarp than in the exocarp.

Diplodia natalensis Pole-Evans produces a postharvest soft rot of citrus fruit that is normally initiated at the stem end. Infection of the button (floral calyx and disk) occurs in the grove prior to harvest, and ingress occurs through the abscission zone as the button abscises (12,14). Postharvest development of soft rot of citrus fruit caused by other fungi is associated with extracellular polygalacturonases (4,6,7). The role of pectolytic enzymes in pathogenesis is well documented (10). Unlike several other postharvest decays of citrus fruit, decay caused by *D. natalensis* is usually not transmitted to sound fruit by contact in packed cartons.

The objectives of the present study were to examine the pectolytic enzymes and cellular changes associated with the decay of Valencia oranges infected with *D. natalensis*. Pectolytic enzyme production by *Penicillium italicum* (Wehmer) was used for comparative purposes. This information is pertinent to understanding the role of pectolytic enzymes in the spread of decay from infected to healthy fruit by contact.

MATERIALS AND METHODS

Mature oranges (*Citrus sinensis* (L.) Osbeck 'Valencia') were inoculated immediately after harvest with mycelium of *D. natalensis* or spores of *P. italicum* introduced through a small incision in the rind as previously described (6). Inoculated fruit

were incubated at 30 and 23 C, respectively, and near 100% relative humidity.

Pectolytic enzymes were extracted by homogenizing decayed rind (mesocarp and exocarp) in 0.1 M tris-(hydroxymethyl)aminomethane + glycine, pH 8.5, containing 2% NaCl. The homogenate was centrifuged at 26,000 g at 0 C for 20 min. Cold acetone (-10 C) was added to the supernatant to 80% and kept at -10 C for 16 hr. The acetone precipitate was collected by centrifugation, dissolved in the extraction buffer, and fractionated by gel filtration on a Sephadex G-100 column equilibrated with the same buffer. Molecular weight was determined by the procedure of Andrews (1). Protein content was determined by the Bio-Rad Laboratories procedure (11).

Exudate from decayed rind was collected by vacuum extraction in a Büchner funnel. The exudate was assayed for polygalacturonase (PG) activity before and after adjustment of the pH with the addition of granular tris-(hydroxymethyl)aminomethane from its initial level of pH 3.6 to pH 8.5.

Polygalacturonase was assayed by measuring the release of reducing groups and by viscosity reduction (6,20); pectin lyase was assayed by the periodate-thiobarbituric acid procedure (3). The substrates used were sodium polypectate (NaPP) and citrus pectin dissolved in 10 mM citrate, pH 5.0, or 50 mM tris, pH 9.1, buffer. Each reaction mixture was incubated at 37 C. The release of alcohol-soluble oligogalacturonic acid was determined as previously described (6). Monogalacturonic acid content in the decayed tissue was determined by the procedure of Fernandez-Flores et al (15).

In vitro production of PG was determined by inoculating orange juice serum medium (OJS) (prepared by centrifuging orange juice) with plugs of mycelium of either *D. natalensis* or *P. italicum*

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growing on potato-dextrose agar. The cultures were incubated on a reciprocating shaker (90 cpm, 3.8 cm amplitude) for 3, 5, and 9 days at 23 C. A series of 3- and 5-day-old cultures was centrifuged to collect the mycelium. The supernatant was autoclaved and again inoculated with mycelial plugs from agar cultures of either *D. natalensis* or *P. italicum* and cultured for an additional 5 days. After the prescribed incubation period, all cultures were centrifuged, and the supernatant was assayed for PG activity. The mycelial pellets were dried in a forced-air oven at 60 C for 24 hr and weighed. The molecular weight of the endopolygalacturonase (endo-PG) produced by *D. natalensis* was determined by extracting the protein with the addition of tris-glycine to 0.1 M, pH 8.5, followed by acetone precipitation and fractionation by gel filtration as described above.

In vitro production of PE by *D. natalensis* was examined by growing the fungus in a mesocarp plus water medium for 5 days at 23 C. PE was assayed by the procedure of Rouse and Atkins (24).

Fresh sections of the rind were taken at the edge of the lesion and from healthy tissue and tested for pectin demethylation (22). Cellular changes were observed within similar tissue by embedding the tissue in plastic (6).

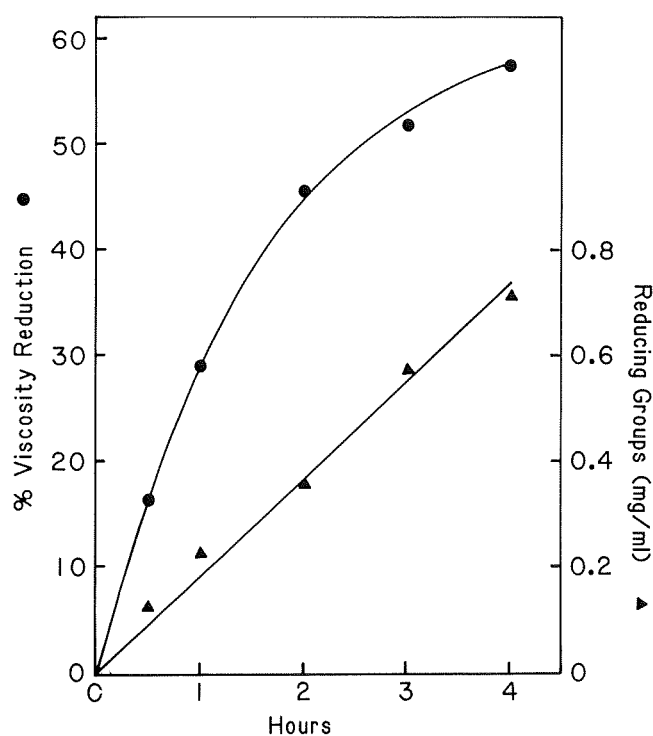


Fig. 1. Relationship between percentage viscosity reduction and release of reducing groups (milligrams per milliliter) during the degradation of sodium polypectate (7.2 mg/ml at pH 5.0) by endopolygalacturonase at 37 C. The enzyme source was from decayed citrus fruit rind infected by *Diplodia natalensis*.

RESULTS

The pectolytic enzyme in the initial extract from decayed tissue infected by *D. natalensis* actively degraded NaPP, and the optimum pH for enzyme activity was pH 5.0. At 50% reduction in viscosity of a NaPP solution (7.2 mg/ml), ~ 484 μ g of reducing groups per milliliter were produced (Fig. 1). The alcohol-soluble oligogalacturonic acid content after 11 hr of reaction time was negligible. Minimal degradation of highly methylated citrus pectin was observed. Degradation characteristics of the pectolytic enzyme were typical of an endo-PG. The initial extract did not exhibit pectolytic enzyme activity on pectin plus 10^{-3} M CaCl_2 at pH 9.3. Fractionation by gel filtration of the initial extract at pH 8.5 revealed the presence of two endo-PG isozymes with molecular weights of 64,000 (endo-PG I) and 45,000 (endo-PG II) daltons and specific activities of 7.11 and 11.46 μ g of reducing groups liberated per microgram of protein per minute, respectively. The specific activity of the initial extract was 1.45. Polygalacturonase activity was not detected in injured-uninfected tissue.

An average of 3.3 mg/gm fresh weight of monogalacturonic acid was found in tissue decayed by *D. natalensis*. Monogalacturonic acid was not detected in nondecayed tissue.

Endo-PG activity in lesions formed by *D. natalensis* was considerably less than in comparable rind tissue infected by *P. italicum*. Approximately 49 ng of protein from tissue decayed by *D. natalensis* produced an equivalent level of endo-PG activity as did 1.5 ng of protein from tissue decayed by *P. italicum*. Minimal endo-PG activity was detected in the exudate from tissue decayed by *D. natalensis*. Less than 5.7% reduction in viscosity was obtained in 1 hr from 500 μ l of exudate. In contrast, 100 μ l of exudate from tissue decayed by *P. italicum* produced almost 90% reduction in viscosity in 15 min. However, adjustment of the pH of the exudate from tissue decayed by *D. natalensis* from pH 3.6 to 6.0, 8.0, and 8.5 prior to assay for percent viscosity reduction of NaPP solution (pH 5.0, 37 C, for 3 hr) substantially increased the endo-PG activities from 8.3 to 13.2, 21.6, and 28.5, respectively.

In vitro production of endo-PG by *D. natalensis* is shown in Table 1. Seventy-eight percent of the endo-PG activity was produced within the first 3 days of a 9-day growth period. However, at 3 days, only 44.5% of the total mycelial growth was attained. Media prepared from a 3- or 5-day-old culture produced growth following reinoculation comparable to the initial culture at 3 days, but only 11% of the endo-PG activity. A 5-day-old culture of *P. italicum* treated in the same manner produced about 81% of its original endo-PG activity, but mycelial growth was less. Endo-PG activity in the culture of *D. natalensis* was associated with a single endo-PG with a molecular weight of 38,000 daltons. *D. natalensis* growing on a medium consisting of mesocarp tissue did not produce PE.

Tissue at the lesion edge of a fruit infected by *D. natalensis* had slightly swollen cell walls and collapsed cytoplasm associated with intercellular hyphae. Most of the pectin in this area was demethylated. Uninfected tissue showed extensive staining for pectin methylation. Maceration at the lesion edge was more evident in the mesocarp than in the exocarp.

TABLE 1. Growth (dry weight) and endopolygalacturonase production by *Diplodia natalensis* and *Penicillium italicum* in orange juice serum^a and the effect of culture age on subsequent endo-PG production

Organism	First growth period			Second growth period ^b		
	Days of growth	Mycelium (mg)	Endo-PG activity ^c	Days of growth	Mycelium (mg)	Endo-PG activity
<i>D. natalensis</i>	3	385 \pm 49 ^d	31.8 \pm 8.9 ^d	5	363 \pm 46	3.7 \pm 2.2
	5	668 \pm 81	37.0 \pm 15.2	5	310 \pm 45	4.1 \pm 1.6
	9	856 \pm 114	41.0 \pm 1.5
<i>P. italicum</i>	5	613 \pm 186	36.3 \pm 4.4	5	405 \pm 142	29.3 \pm 8.4

^aCultures were incubated at 23 C on a reciprocating shaker (90 cpm, 3.8 cm amplitude).

^bCultures from the first growth period were centrifuged to remove mycelium, autoclaved, and inoculated with mycelium of the respective organism.

^cPercentage viscosity reduction of a sodium polypectate solution, pH 5, at 37 C in 1 hr.

^dMean and standard deviation of six replications.

DISCUSSION

The decay of citrus fruit by *D. natalensis* was found to be associated with two isozymes of endo-PG, which were presumably produced by the fungus. *D. natalensis* was shown to produce in vitro an endo-PG with a molecular weight slightly smaller than the endo-PG II isozyme recovered from decayed tissue. Riou (23) reported finding PG activity in citrus fruit, but the extracts showed an extremely low level of activity. Our findings indicate that endo-PG is not produced by the fruit in response to an injury. A pH of 5.0 for optimum endo-PG activity in vitro corresponds favorably to the pH of the partially macerated tissue which was near pH 4.2 at the edge of the lesion. The molecular weights of the two endo-PG isozymes were within the range of endo-PG isozymes produced by other phytopathogenic fungi (9,21).

The presence of monogalacturonic acid in the decayed tissue did not indicate that an exo-PG also was produced in vivo. Purified endo-PG preparations have been reported to utilize both oligo- and polygalacturonic acid as substrates for the direct formation of monogalacturonic acid (5,19). Apparently, in vivo conditions favor a more complete degradation of pectin by endo-PG than do in vitro conditions.

An apparent role of extracellular endo-PG is to cause sufficient pectin degradation to permit intercellular penetration by hyphae of *D. natalensis* and other fungi causing soft rot of citrus fruit (6,7). The level of endo-PG associated with the decay process apparently is not an important factor in determining the rate of decay. Decay caused by *D. natalensis* develops much more rapidly than decay caused by *P. italicum* (14) even though endo-PG activity is considerably less in rind infected by *D. natalensis*. However, rind infected by *D. natalensis*, especially at the edge of the lesion, is firmer than comparable tissue infected by *P. italicum*. This difference in firmness could be partially explained by a low level of endo-PG in tissue infected by *D. natalensis* and from the observation that tissue maceration was more evident in the mesocarp than in the exocarp at the lesion edge.

The low level of endo-PG detected in decayed rind infected by *D. natalensis* could possibly be due to a repression of endo-PG synthesis. In vitro production of endo-PG by *D. natalensis* did not parallel the growth of *D. natalensis*. In addition, endo-PG production by *D. natalensis* growing in an OJS medium, which previously had supported 3 or 5 days of growth of *D. natalensis*, was repressed. Endo-PG production by *P. italicum* was not influenced by similar culture conditions. The nature or origin of the in vitro repression factor is not known. Catabolic repression of cell-wall-degrading enzyme synthesis during pathogenesis has been reported (13,27).

In addition to a low level of endo-PG associated with the decay of citrus fruit by *D. natalensis*, endo-PG in the exudate of this same tissue was minimal. In contrast, endo-PG activity in the exudate from tissue decayed by *P. italicum* was very high. Interestingly, increasing the pH of the exudate from tissue decayed by *D. natalensis* from pH 3.6 to pH 8.5 prior to the assay resulted in the detection of a higher level of endo-PG activity. This effect may indicate that endo-PG in the exudate was inactivated. Fielding (16) reported that low PG activity in decayed tissue of plum and peach fruit infected by *Monilinia fructigena* was due to inactivation of the enzyme by a proteinlike compound present only in the decayed tissue. Proteinaceous inhibitors of PG have been identified by others (2,17). If an inhibitor is present, it apparently has minimal effect on the decay process based on the rate of decay development and the extent of tissue maceration. The near absence of endo-PG activity in the exudate which accumulates at the contacting surfaces of a decayed and uninfected fruit could be an important factor in restricting the spread of *D. natalensis* by contact. Schiffmann-Nadel (26) has suggested that pectolytic enzymes may be important in damaging the peel and creating a site for hyphal penetration. *Penicillium italicum* is readily transmitted to an uninfected fruit by contact (8), and the exudate contains high endo-PG activity.

The demethylation of pectin in cell walls at the edge of the lesion caused by *D. natalensis* has also been observed in comparable tissue decayed by both *P. italicum* and *P. digitatum* (6,7).

Demethylation is attributed to the endogenous PE, since citrus peel does contain PE (25). In addition, *D. natalensis* growing in a liquid medium of citrus fruit mesocarp tissue was not found to produce PE. The demethylation process would aid pectin degradation by endo-PG, since the enzyme randomly cleaves the pectin chain at de-esterified sites (18).

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