Reduced Polygalacturonase Activity in dsRNA-Containing Hypovirulent Strains of Cryphonectria parasitica

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ABSTRACT. The production of extracellular polygalacturonase (PG) by virulent and dsRNA-containing hypovirulent strains of *Cryphonectria parasitica* was studied both in diseased tissues and *in vitro* culture using minimal medium with sodium polypectate as the substrate. In liquid minimal medium, PG of virulent strains attained maximum activity after about 10 days of incubation, which coincided with maximum mycelial growth. The isogenic dsRNA-containing hypovirulent strains were generally lower in PG activity. Four virulent and 10 dsRNA-containing strains were screened for PG production on a medium containing 0.5% polygalacturonic acid, 0.2% sucrose, 0.2% ammonium sulfate and 1.5% agar. Polygalacturonase activity generally correlated well with fungal virulence when tested on American chestnut stems. A modified "cup-plate" assay was used to test the PG activity in bark taken from the margin of cankers. Bark from cankers caused by virulent strains were generally higher in PG activity, compared to bark from cankers caused by dsRNA-containing hypovirulent strains. PG isozymes were detected in culture filtrates by native polyacrylamide gel electrophoresis (PAGE) and by isoelectric focusing (IEF) with an activity stain overlay technique. A single band was detected with PAGE of native proteins while two isozymes were detected by IEF with PIs at approximately 4.1 and 4.5.

Cyphonectria parasitica (Murr.) Barr is the causal fungus of chestnut blight, a canker disease that annihilated the American chestnut in less than fifty years (15). The mechanisms by which the fungus colonizes host tissue, and advances into the sound tissue have been described (4, 14, 18),

but many questions about the pathogenesis of this fungus remain unanswered. The discovery of cytoplasmically transmissible, dsRNA-containing hypovirulent strains of *C. parasitica* and the association of hypovirulence with the recovery of European chestnut from blight have stimulated a search for factors affecting the expression of virulence. Oxalic acid was suggested to play an important role in the pathogenicity of *C. parasitica* by chelating the calcium in the pectin of cell wall middle lamellae and thereby exposing it to greater degradation by pectic enzymes (19). Reduced oxalic acid production by some hypovirulent strains has been reported (12), however, Bennett and Hindal (1, 2) found no consistency between oxalic acid production and virulence. Studies have shown the association of dsRNA in hypovirulent strains with down-regulation of many fungal polypeptides (9), including some with enzyme activity (6, 13). To date, none of these polypeptides has been found to be responsible for the reduced virulence.

Cell wall-degrading pectic enzymes are produced by fungal pathogens in many fungal-plant interactions (7). Polygalacturonase (EC 3. 2. 1. 15) (PG) has been suggested as one of these important enzymes required for the expression of virulence (10, 17). This study was undertaken to test the production of PG by both virulent and their isogenic dsRNA-containing hypovirulent strains *in vitro* and *in vivo* to determine its possible role in the pathogenesis of *C. parasitica.*

MATERIALS AND METHODS

C. parasitica strains and culture conditions. Two virulent strains, Ep155 (ATCC No. 38755) and Ep523 (ATCC No. 66022), and their isogenic hypovirulent strains that contain dsRNA of I1 (Ep779, Ep395) and M2 (Ep868, Ep524) (11) were grown in 10 ml of liquid minimal medium (21) in 125 ml flasks, with 1% sodium polypectate as the sole carbon source. The initial pH of the medium was adjusted to 5.0. The inoculum consisted of 4 mycelial plugs (6-mm-diameter) taken from the edge of each culture. Cultures were incubated stationary at 25 C with a 16-h photoperiod for up to 14 days.

Preparation of culture filtrates and PG activity assay. Culture filtrates were collected every 2 days and their pH was determined. Mycelium was collected by centrifugation at 25,000 g for 15 min at 4 C and its dry weight recorded after oven drying at 80 C for 24 h. PG activity was assayed by the reducing sugar method (8) and the revised "cup-plate" diffusion assay (25). Both methods gave similar results, but the latter was easier to manipulate. For the diffusion assay, 0.75-mm thick agarose gels were cast on 18 x 20 cm slab gel casting plates. The agarose solution contained 1% agarose, 0.1% polygalacturonic acid, and 10 mM EDTA in 0.1 M sodium acetate (pH 5.3) buffer. The gel was cast in the gel mold pre-heated to 50 C. Cork borer (6-mm-diameter) wells were filled with 20 μl of culture filtrate or standard enzyme. In each assay plate a commercial PG from *Rhizopus* spp. (Sigma, St. Louis, Mo.) was used to construct a standard curve ranging from 1.25 to 20 x 10^{-3} units/ml. The assay plate was incubated at 25 C for 24 h in a moist chamber. The gel was developed after incubation by flooding it with 10 ml of 0.05% ruthenium red (Sigma) for 20 min, and then rinsed with deionized water. The average diameter of each clear zone, determined by two measurements at right angles, was used to calculate the PG activity from the standard curve. The diameter of the clear zone was proportional to the logarithm of PG activity.

A 12-day-old culture filtrate of Ep155 was used for polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing (IEF) of native protein. The culture filtrate was dialyzed against double deionized water for 2 days at 4 C and lyophilized. The amount of protein in samples concentrated 100-fold was measured by the method of Bradford (3) with bovine serum albumin (Sigma) as the standard.

PG activity of fungi growing on an artificial medium or in diseased bark. The artificial medium consisted of 0.5% sodium polypectate, 0.2% sucrose, 0.2% ammonium sulfate and 1.5% agar (PGA medium) (24). Plugs of mycelium were taken from the edge of virulent and hypovirulent colonies (Table 1) and placed on PGA medium. After 2 days of incubation at 25 C, plates were flooded with 5 ml of 5 N HC1 for 5 min, and then rinsed with deionized water. The size of the clear zone around the fungal colony was related to PG activity. PG activity in cankered bark tissue was assayed by the modified Dingle et al. (9) "cup-plate"

Table 1. Strains of *Cryphonectria parasitica* assayed for polygalacturonase (PG) activity on PGA medium* and virulence on dormant American chestnut stems.

Strain	ATCC #	dsRNA	PG activity†	Vir.‡
Ep155	38755	–	++++	++++
Ep154		–	++++	+++
Ep42	38751	–	++++	+++
Ep523	66022	–	++++	++++
Ep154I		+	+	–
Ep42I	52573	+	+	–
Ep47	38760	+	+	++
Ep63		+	++	+
Ep405		+	++++	+++
Ep713	52571	+	++	–
Ep868		+	++	++
Ep779		+	++	++
Ep524	66023	+	++	++
Ep395	66020	+	++	++

*PGA medium consisted of 0.5% polygalacturonic acid, 0.2% sucrose, 0.2% ammonium sulfate and 1.5% agar.

†PG activity on PGA medium: ++++ the most PG activity, clear zone diameter is about twice that of the seeded mycelial plug; +++ clear zone diameter is equivalent to the seeded mycelial plug; ++ clear zone diameter is half of the seeded mycelial plug; + the clear zone is not obvious.

‡Virulence expressed by the canker size on dormant American chestnut stem segments 40 days after inoculation except for Ep523, Ep868, Ep779, Ep524 and Ep395 that were from the results of Elliston (11). ++++ Canker size is 80–100% of Ep155; +++ Canker size is 50–79% of Ep155; ++ Canker size is 20–49% of Ep155; + Canker size is <20% of Ep155; – No canker formed.

assay. The medium contained 1% sodium polypectate in 0.1 M sodium acetate buffer (pH 5.3), 0.5% ammonium oxalate, and 1.5% agar. Plugs of the agar medium were removed with a cork borer and refilled with bark discs of the same size taken from the margin of a canker. The plates were incubated at 25 C for 36 h and then developed with 5 N HC1 as above.

The virulence of each strain was assessed by inoculation of excised dormant American chestnut stems (23), as well as by previously published data (11).

Activity stain for PG after native PAGE and IEF. The native PAGE was performed using the system of Laemmli (16) that employed 0.75 mm gels in a vertical slab unit (Mini-protein II, Bio-Rad, Richmond, Calif.) with a resolving gel containing 10% acrylamide and 0.33% bisacrylamide. The stacking gel contained 5% acrylamide and 0.17% bisacrylamide. Concentrated culture filtrates were electrophoresed at 4 C and 200 V until the tracking dye migrated to the bottom of the gel. After electrophoresis, the native gel was treated with two 30 ml changes of 0.1 **M** sodium acetate buffer (pH 5.3) for 30 min with shaking. A 0.4-mm thick overlay agarose gel cast on a GelBond support film (FMC Bioproducts, Rockland, Maine), containing 0.1% polygalacturonic acid in 0.1 M sodium acetate buffer (pH 5.3) and 10 mM EDTA, was placed on the resolving gel and incubated for 6 h in a moist chamber at 25 C. The overlay gel was stained with 0.05% ruthenium red for 20 min and rinsed with deionized water for the detection of bands with PG activity.

The IEF was performed according to the method of Ried and Collmer (22) with modifications. Both the IEF polyacrylamide gel and the agarose overlay gel were 0.4 mm thick. Electrofocusing was performed at 6 C on an LKB Multiphor II apparatus (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ.). The gel was pre-electrofocused for 15 min at 50 V. Samples containing 0.5 to 2.5 μg total protein were applied directly to the gel as a series of drops. The gel was then focused at constant 0.5W with a maximum of 500 V for about two-and-one-half h. After focusing was completed, the pH of the gel was determined directly with a flat electrode. The overlay agarose gel was developed as above after 2 h of incubation.

RESULTS

Production of PG activity. Culture filtrates from virulent and isogenic hypovirulent strains differed in PG activity over time (Figure 1). The two virulent strains reached maximal PG activity after about 10 days of incubation, which is when maximal mycelial growth occurred (Figure 2). The PG activity of two hypovirulent strains that contained dsRNA M2, Ep868 and Ep524, was about one-third of their isogenic virulent strains. The PG activity of hypovirulent strains containing dsRNA I_t, Ep779 and Ep395, showed no clear difference from their isogenic virulent strains in the first 6 days of incubation; however, PG activity decreased dramatically after 8 days of incubation and was not detectable by the diffusion method at day 14. This decrease of PG activity seems correlated with the

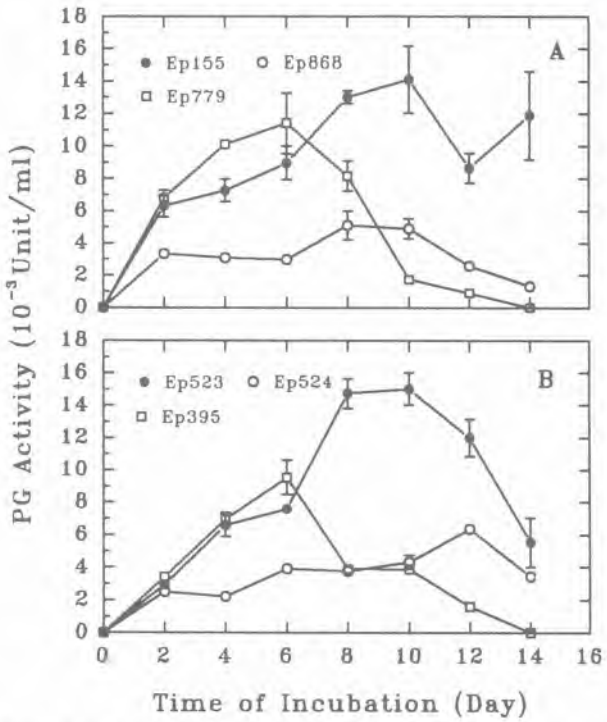

Figure 1. Polygalacturonase activity at different incubation times in culture filtrates of *Cryphonectria parasitica* virulent strains Ep155 and Ep523, and isogenic hypovirulent strains that contain dsRNA designated as M_2, Ep868, Ep524, or I_1, Ep779, Ep395, respectively. Vertical bars represent standard errors of means of 3 replicates.

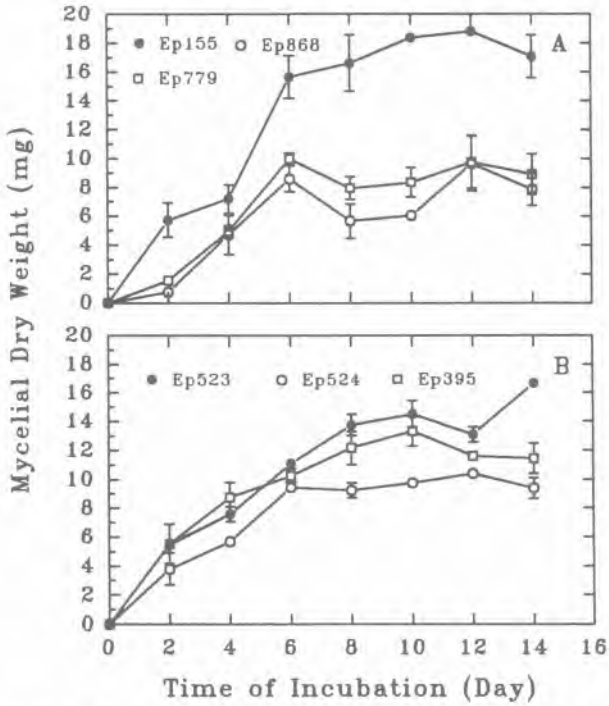

Figure 2. Mycelial growth of *Cryphonectria parasitica* virulent strains Ep155 and Ep523, and isogenic hypovirulent strains that contain dsRNA designated as M_2, Ep868, Ep524, or I_1, Ep779, Ep395, respectively. Vertical bars represent standard errors of means of 3 replicates.

increase of pH in the cultures of hypovirulent strains Ep779 and Ep395 (Figure 3). The pH values of all the cultures dropped about 0.25 unit in the first 2 days of incubation, and then started to increase as the culture aged. Cultures of the two hypovirulent strains containing dsRNA (Ep779 and Ep395) increased the most in pH (about 1.25 units), whereas the virulent strains (Ep155 and Ep523) and the other two hypovirulent strains containing dsRNA M2 (Ep868 and Ep524) increased only about 0.25 to 0.50 unit.

The relatedness of PG activity and virulence. In the PGA medium assay, PG activity was correlated with the size of the clear zone (Figure 4). The PG activity of virulent and hypovirulent strains assayed in PGA medium, as well as their relative virulence are summarized in Table 1. The PG activity of most strains was closely related to their virulence. This relationship also was shown when the PG activity was assayed in cankered bark tissue (Figure 5). Strains that caused large cankers were high in PG activity, whereas those that caused small cankers were lower in PG activity.

Activity stain for PG after native PAGE and IEF. One PG isozyme was detected by native PAGE and the activity stain overlay technique (Figure 6). However, on IEF gels, two isozymes of PG with PIs of approximately 4.1 and 4.5 were detected (Figure 7). Both methods detected as little as 0.6 ug total protein. PG activity was not detected by native PAGE in cultures with glucose as the sole carbon source. PG apparently is an induced enzyme.

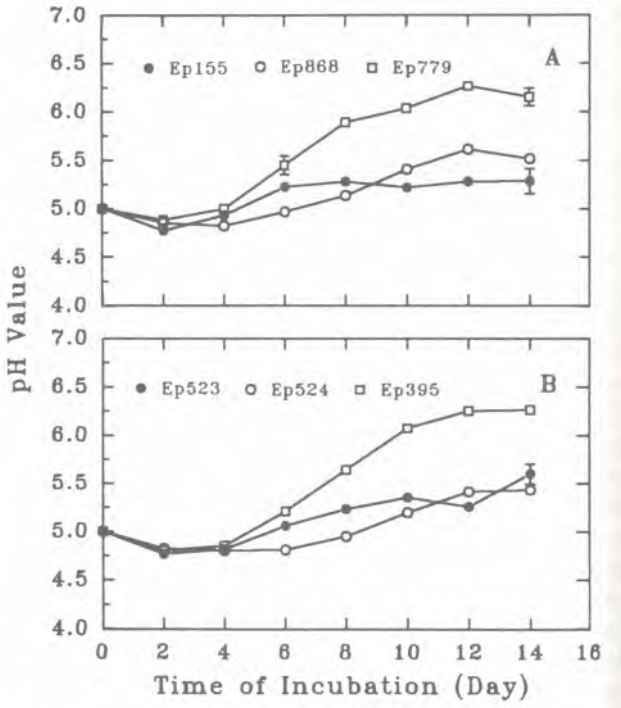

Figure 3. Change in pH in culture filtrates of *Cryphonectria parasitica* virulent strains Ep155 and Ep523, and isogenic hypovirulent strains that contain dsRNA designated as M_2, Ep868, Ep524, or I_1, Ep779, Ep395, respectively. Vertical bars represent standard errors of means of 3 replicates.

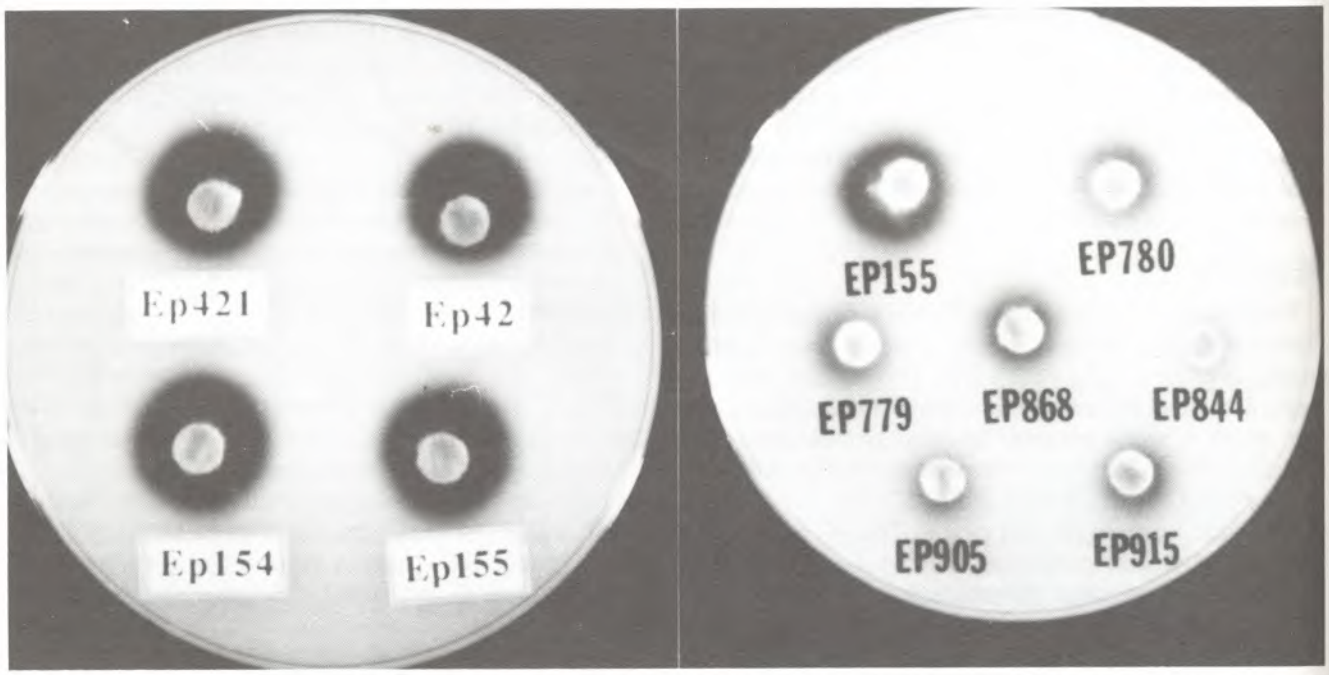

Figure 4. Polygalacturonase activity assayed on PGA medium (24) that contains 0.5% polygalacturonic acid, 0.2% sucrose, 0.2% ammonium sulfate and 1.5% agar. Virulent strains of *Cryphonectria parasitica*: Ep421, Ep42, Ep154, Ep155. Hypovirulent strains: Ep780, Ep779, Ep868, Ep844, Ep905, Ep915 are isogenic to Ep155.

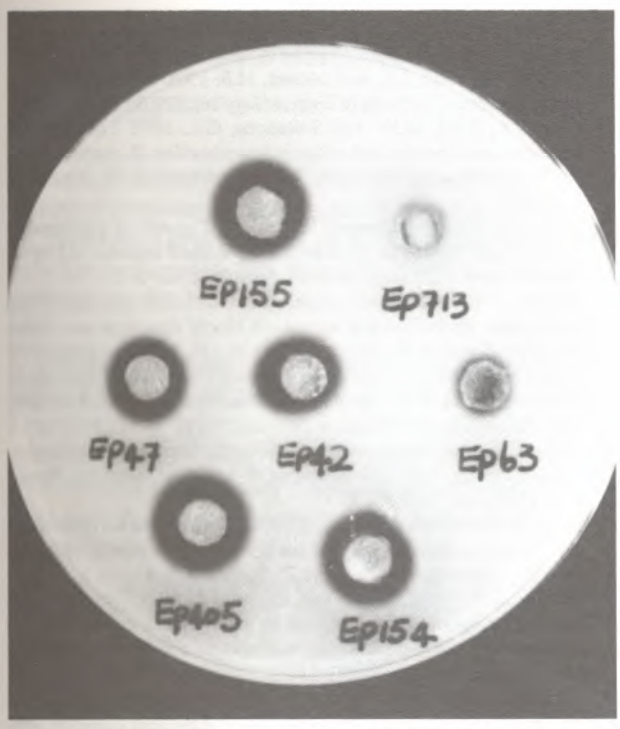

Figure 5. Polygalacturonase activity produced in the cankered American chestnut bark infected with virulent strains of *Cryphonectria parasitica* (Ep155, Ep42, Ep154), and hypovirulent strains (Ep405, Ep47, Ep713, Ep63).

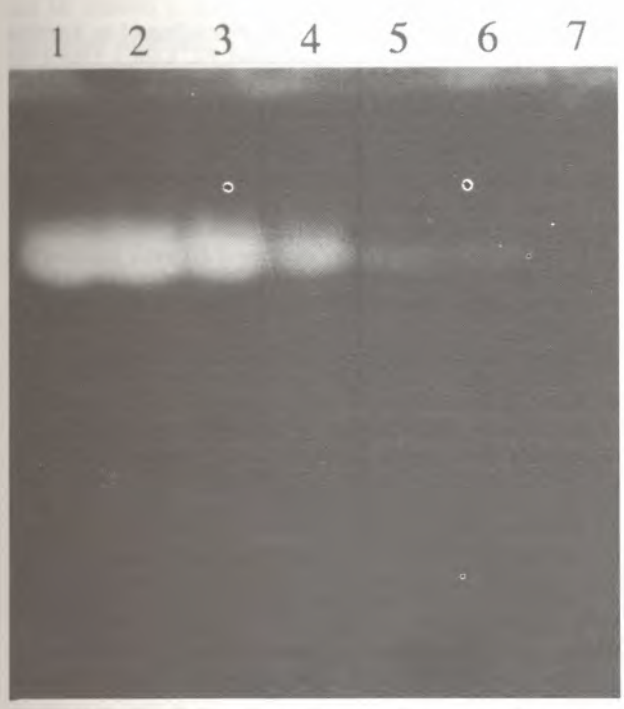

Figure 6. Activity stain for polygalacturonase after native polyacrylamide electrophoresis. Samples were from culture filtrate of virulent strain of *Cryphonectria parasitica* Ep155, concentrated about 100 fold by lyophilization. Lanes 1, 2, 3, 4, 5, and 6 were 10, 8, 4, 2, 1 and 0.6 μg of protein respectively. Lane 7 was 0.6 μg of protein from culture filtrate with glucose as the carbon source.

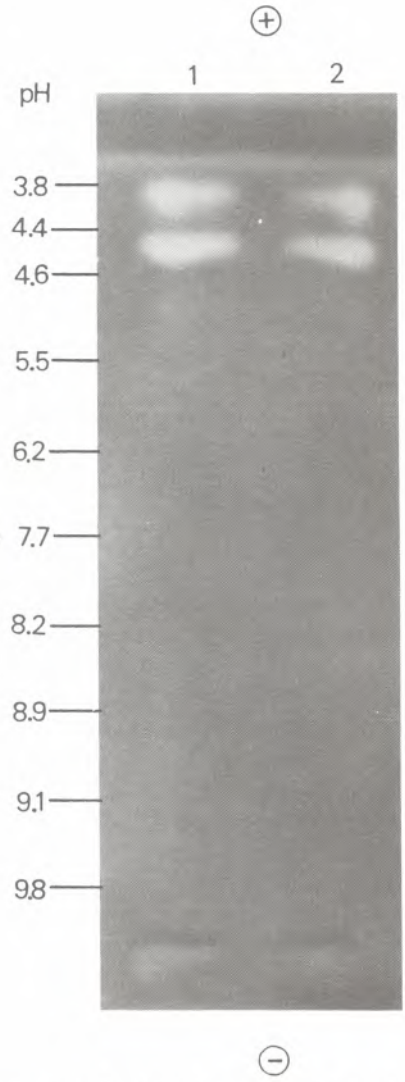

Figure 7. Activity stain for polygalacturonase after isoelectric focusing. Samples were from culture filtrate of virulent strain of *Cryphonectria parasitica* Ep155, concentrated about 100 fold by lyophilization. Lane 1: 2.5 μg of protein; Lane 2: 1.5 μg of protein.

DISCUSSION

This preliminary study suggests that PG, an important cell wall-degrading enzyme produced by many pathogenic fungi, is induced at different levels among virulent and dsRNA-containing hypovirulent strains of *C. parasitica.* The decreased PG activity in most hypovirulent strains seems correlated with their reduced virulence. The *in vivo* "cup-plate" assay also showed that hypovirulent strains that caused smaller cankers produced less PG activity within cankered bark tissues. Differences in the time course of PG production by hypovirulent strains may be a reflection of their differing dsRNA. Studies have shown that dsRNA could affect the expression of many fungal products (5, 6), including enzyme activity (13). Reduced mycelial growth may contribute to the lower expression of different enzymes, but some hypovirulent strains were comparable in growth rate to their isogenic virulent strains, but were lower in PG activity. The increase of pH in cultures of dsRNA I1-containing strains may account for the decrease of PG activity; a previous study (20) suggested that pH 5.3 is about optimal for PG activity. Other possible explanations for the observed reduction in PG activity could be related directly to the expression of dsRNA I_1. In the PGA medium assay, the dsRNA Ii-containing strains were lower in PG activity even in the first 2 days of culture, while in liquid medium, a reduction was seen only when the culture aged. A possible explanation for this is that the inhibitory factor(s) expressed by the dsRNA II was not as well diffused in solid agar medium as compared to the liquid medium. A further study to confirm this is needed. Although PG activity and virulence were closely related in this study, a more detailed analysis of the function of PG during the pathogenicity of *C. parasitica* is necessary. The relative simplicity of 2 PG isozymes may facilitate enzyme purification and subsequent molecular cloning of the PG genes. Then, the function of PG may be elucidated by gene disruption (24).

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