Hydrolytic Activity in Supernatant from Germinated Spores of Venturia inaequalis

E. D. Pellizzari, J. Kuć, and E. B. Williams

Department of Biochemistry and Department of Botany and Plant Pathology, Purdue University, Lafayette, Indiana 47907.

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Extracellular enzymes produced by *Venturia inaequalis* (Cke.) Wint. in culture filtrates may have a role in pathogenesis (5, 10). The production of phosphatidases by other fungi have been detected in culture filtrates (8, 12, 13). Plant cell membranes contain phospholipids (2) and the production of phosphatidases by phytopathogenic organisms could be related to changes in host cell permeability during pathogenesis (7, 8, 11). This study reports the finding of hydrolytic activity in supernatants from germinated spores of races 1 through 5 of *V. inaequalis* on the substrates *p*-nitrophenyl acetate, *p*-nitrophenyl laurate, and DL-α-lecithin dipalmitoyl.

Spore suspensions of *V. inaequalis* were prepared as described by Barnes & Williams (1). These were centrifuged twice, and each time resuspended in 10 ml of 0.05 M phosphate buffer (pH 7.0). The concentrations of spores were determined on a microscopic grid (Petroff-Hausser bacteria counter, C. A. Hauser & Son, Phil., Pa.). Spore suspensions from isolates representing the five races were individually incubated at 19 C for 48 hr. Supernatants of germinated spores were obtained by centrifuging at 2,000 *g*, and these were dialyzed against 6 liters of deionized water for 18 hr at 4 C. Each supernatant was incubated at 25 C in the presence of *p*-nitrophenyl phosphate (NPP), *p*-nitrophenyl acetate (NPA), or *p*-nitrophenyl laurate (NPL). The method of Garen & Levinthal (3) was modified to determine phosphatase activity. The reaction mixture for the phosphatase assay consisted of 2.0 ml of dialyzed supernatant, an equal volume of 1 M Tris buffer (pH 7.5), and 1.0 ml of 0.001 M *p*-nitrophenyl phosphate in the same buffer. The NPA and NPL assays were adapted from the procedures described by Huggins & Lapides (6). The reaction mixtures for the NPA and NPL assays consisted of 2.0 ml of dialyzed supernatant, an equal volume of 0.05 M phosphate buffer (pH 7.0), and 1.0 ml of NPA or NPL solution. The NPA and NPL solutions were prepared by dissolving 65 mg of substrate in 10 ml of absolute methanol and diluting 50-fold with 0.05 M phosphate buffer. Supernatants from germinated spores were autoclaved at 15 psi and 121 C for 30 min, and served as controls. Hydrolysis of *p*-nitrophenyl derivatives yield *p*-nitrophenol (NP) that can be measured spectrophotometrically at 400 mμ. Supernatants from the five races were capable of hydrolyzing NPA (9) and NPL, but were unable to hydrolyze NPP (Fig. 1 A, B, C, D, E). Differences in the rate of hydrolysis may be attributed to variation in spore concentrations, isolates, or races, or may reflect differences in the affinity for NPL.

The method of Hills & Mottern (4) was modified to measure pectin methyl esterase (PE) activity in supernatants from germinated spores. The decrease in pH from liberation of carboxyl groups in a citrus pectin solution was continuously recorded with a Beckman potentiometric recorder (Model No. 93506) attached to a radiometer pH meter 22. A pH range of 5 to 7 was adjusted to full scale on the recorder, using standard buffers and a variable 10 kohm potentiometer. The substrate for the PE assay consisted of 0.5% (w/v) citrus pectin (Nutritional Biochemical Corp.) dissolved in 0.1 M NaCl solution and adjusted to pH 6.8 with 0.02 N NAOH. Commercial pectin methylesterase (Nutritional Biochemical Corp.) was dissolved in 10% (w/v) NaCl, and 1.5 units of PE activity were added to 25 ml of substrate at 25 C. The incubating medium was continuously stirred, and the decrease in pH fol-

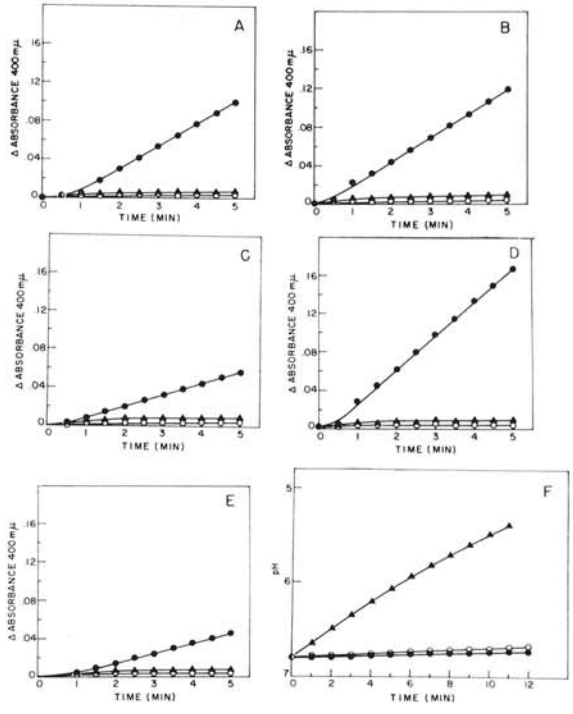

Fig. 1. Hydrolytic activity of supernatants 48 hr after germination of *Venturia inaequalis* spores in 0.05 M phosphate buffer (pH 7.0). A through E depict assays using *p*-nitrophenyl laurate (NPL) with supernatant (solid circles), *p*-nitrophenyl phosphate (NPP) with dialyzed supernatant (open circles), and NPL with autoclaved supernatant (triangles). **A)** Race 1 (isolate No. 1752-1) supernatant from 10^8 spores ml. **B)** Race 2 (isolate No. 839-6), 8×10^7 spores/ml. **C)** Race 3 (isolate No. 1140-6), 3×10^7 spores/ml. **D)** Race 4 (isolate No. 1743-4), 2×10^7 spores/ml. **E)** Race 5 (isolate No. 1763-4), 10^7 spores/ml. **F)** Effect of 1.5 units of commercial PE (triangles), race 1, 2, 3, 4, or 5 supernatants (solid circles), and water control (open circles), respectively, on pH in a medium containing citrus pectin.

TABLE 1. Liberation of palmitic acid from DL-α-lecithin dipalmitoyl by activity in supernatant from germinated spores of *Venturia inaequalis*

Reaction mixture[a]	μg palmitic acid detected[b]
Lecithin, — supernatant	1.04[c]
Lecithin, + boiled supernatant	1.13
Lecithin, + supernatant	2.13
Lecithin, 2 × supernatant[d]	4.02

[a] Reaction mixture incubated for 24 hr at 30 C.
[b] Amount calculated from standard curve for methyl palmitate (9).
[c] Average values from duplicate experiments.
[d] Volume of supernatant added to the reaction mixture was doubled.

lowed (Fig. 1-F). A 2-ml aliquot of dialyzed supernatant from germinated spores of each race (Fig. 1-A-E depict the activities of these supernatants using NPL as a substrate) was added to 25 ml of pectin solution. The pH of the pectin solution remained constant for all supernatants studied (Fig. 1-F). Commercial PE representing 15 units of activity on pectin was assayed with NPA and NPL as described above. Pectin methyl esterase did not hydrolyze NPA or NPL. These results suggest that the activity(s) in supernatant from germinated spores is not PE.

Activity in spore supernatants was assayed with lecithin. Supernatant was prepared from a mixture of germinated spores (races 1 through 5) as described earlier. The assay medium contained 2.75 μM DL-α-lecithin dipalmitoyl (Nutritional Biochemical Corp.) and 1.0 ml of spore supernatant in 0.05 M phosphate buffer (pH 7.0). Controls consisted of substrate in 1.0 ml of buffer and substrate added to 1.0 ml of supernatant previously boiled for 10 min. Each medium was incubated and extracted for free palmitic acid followed by methylation of the fatty acid according to the methods of Tseng & Bateman (13). Methylated samples were dissolved in 50 μliters of hexane containing 1.55 μg/μliter of heptadecane as an internal standard. Analyses of samples were performed on a Hewlett-Packard instrument (F & M, model 810-DR-12) equipped with a stainless steel column and flame ionization detector. Columns (one-eighth inch × six ft long) were packed with Chromosorb Q (100/120 mesh) coated with 3% HiEFF-3BP (Applied Science Co.). Carrier gas (He), H_2, and air flow rates were 45, 43, and 485 ml/min, respectively. Injection port and detector temperatures were 220 C. The column temperature was held constant at 150 C for 1 min, and then programmed 15 degrees/min to 180 C and maintained at the upper limit for 2 min. The amount of methyl palmitate detected in each sample was obtained from a standard curve employing the internal standardization method (9). Supernatants from a mixture of germinated spores were capable of liberating palmitic acid from DL-α-lecithin dipalmitoyl (Table 1).

Supernatants from germinated spores hydrolyzed NPA, NPL, and DL-α-lecithin dipalmitoyl, but were unable to hydrolyze pectin or NPP. It is concluded that the hydrolytic activity is not due to a phosphatase or PE.

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