

Phospholipase activity in cultures of *Phytophthora infestans* and in infected potato leaves

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Phospholipase B activity was detected in liquid and agar cultures of *Phytophthora infestans* and in infected potato leaves. The phospholipase activity in culture filtrates was stimulated 15-fold by deleting glucose from liquid rye medium. An additional 35-fold stimulation occurred after adding PC to the cultures. Much less phospholipase activity was found when the fungus was grown on liquid V/8 medium, and no phospholipase activity was detected with liquid pea, bean or synthetic media, even after the addition of PC. The PC-stimulated rye culture filtrates exhibited not only phospholipase activity, but also galactolipase and acylglycerol lipase activities. The apparent K_m for PC (dipalmitoyl) was 2.86 μM . The phospholipase activity from fungal cultures exhibited optimal activity at pH 9.0 and was strongly inhibited by DTT. During the infection of potato leaves there was a large (more than 14-fold) increase in phospholipase activity. The phospholipase activity in infected leaves had a major peak of activity at pH 9.0, which was inhibited by DTT and a minor peak of activity at pH 6.0 which was insensitive to DTT.

INTRODUCTION

Phospholipase activity has previously been detected in cultures of several species of phytopathogens using the cup-plate assay technique [18]. This technique is incapable of distinguishing whether the phospholipase activity is type A₁ (E.C.3.1.1.32), A₂ (E.C.3.1.1.4), B (E.C.3.1.1.5), C (E.C.3.1.4.3) or D (E.C.3.1.4.4). With other analytical techniques phospholipase B has been detected in culture filtrates of *Sclerotinia sclerotium* [12], *Sclerotium rolfii* [18], *Thielaviopsis basicola* [11], *Botrytis cinerea* [17] and *Phoma medicaginis* [16]. Phospholipase C has been detected in the culture filtrates of *Erwinia carotovora* [20]. The occurrence of phospholipase B has also been observed during the *in vivo* infection of bean tissue by *T. basicola* [11], *S. rolfii* [19] and *Uromyces phaseoli* [9].

The toxicity of extracellular phospholipases and their role in pathogenesis has been studied only in two host-pathogen interactions. When phospholipase B from *B. cinerea* was purified, it did not lyse protoplasts but caused the leakage of acid phosphatase from a lysosome-enriched fraction of potato sprouts [17]. These observations led the authors to suggest that this phospholipase may traverse the host plasma membrane

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Abbreviations used: BSA, bovine serum albumin; DTT, dithiothreitol; DGDG, digalactosyldiglyceride; MGDG, monogalactosyldiglyceride; PC, phosphatidylcholine; TLC, thin layer chromatography.

and degrade certain intracellular membranes [17]. In *E. carotovora* phospholipase C was purified and found to lyse cucumber protoplasts [20], and a separate endopolygalacturonate trans-eliminase caused maceration, cell death and ion leakage [14]. These studies suggest that phospholipases may play an important role during pathogenic interactions.

In this initial report, we identify the optimal culture conditions for the production of phospholipase activity in liquid cultures of *Phytophthora infestans*. We also present evidence that suggests that the same enzyme activity is found in agar culture and in infected potato leaves.

MATERIALS AND METHODS

Cultures of *P. infestans* (Mont.) de Bary isolates R-OA and R-4A (obtained from W. Fry, Ithaca, NY) were maintained on complete rye steep agar medium [6] at 20°C. Sporangia were harvested after 10–14 days by flushing the surface of the agar with sterile distilled water and lightly scraping with a bent glass rod. Mycelia were removed by filtering the sporangia preparation through two layers of cheesecloth. Filtered sporangia (1×10^6) were then used to inoculate fresh agar or liquid cultures. Liquid cultures were prepared by placing 100 ml of medium in 1000-ml Erlenmeyer flasks covered with aluminium foil or cotton plugs. Liquid cultures were incubated without agitation at 20°C. For liquid cultures, a modified rye steep medium [6] was prepared. Glucose was added only when indicated. Liquid V/8 media was prepared as described [1]. Liquid pea and bean media were prepared by adding 160 g Bird's Eye Peas or 400 g Bird's Eye Lima Beans, respectively, to 1 l of water. The mixtures were each homogenized for 3 min in a Waring Blender and the slurries were filtered through four layers of cheesecloth. Hohl's P-1 synthetic medium was prepared as described [7]. Before being autoclaved (20 min at 121°C), various compounds (Tables 1 and 2, Fig. 2) were dissolved or emulsified in 5 ml of water, added to liquid cultures, and the final pH was adjusted to 5.8.

Freshly isolated cultures (isolates R-1, 2V and R-OV) (obtained from R. Goth,

TABLE 1
Phospholipase activity in 7-day culture filtrates of P. infestans grown on various types of media

Medium		Phospholipase activity (nmol/h/ml)
Rye steep	(+ glucose)	0.4
Rye steep	(- glucose)	6.0
Rye steep	+ 10 mg PC	214
V/8		1.7
V/8	+ 10 mg PC	1.9
Pea		<0.1
Pea	+ 10 mg PC	<0.1
Bean		<0.1
Bean	+ 10 mg PC	<0.1
Hohl's P-1	(includes 20 mg PC)	<0.1

Beltsville, MD) were stored on corn seed media to retain virulence [3]. Infected corn seeds were used to inoculate bean agar [3] in Petri dishes.

Potato plants cv. Katahdin were grown from seed tubers in the greenhouse for 4–6 weeks. Leaves were detached and placed in trays of moist vermiculite. Zoospores were prepared (from isolate R-1, 2V) and leaves were inoculated as described [3]. Trays were covered with parafilm and incubated under fluorescent lights at 20°C. After infection, one leaflet (4–5 cm in length) was removed and ground in 10 ml 0.3 M sucrose, 0.1 M tricine–NaOH (pH 7.0) and 5 mM β -mercaptoethanol. The homogenate was filtered through two layers of cheesecloth and centrifuged at 20 000 g for 30 m. The supernatant was removed and assayed directly for activity.

Enzyme preparation

For the analysis of extracellular enzyme activities, the cultures were swirled vigorously for 30 s, and an aliquot of medium was removed and filtered through Whatman No. 1 filter paper to remove mycelia and sporangia. This culture filtrate was used directly as a source of enzyme for most experiments.

For the analysis of intracellular enzyme activities a 7-day mycelial mat was collected on filter paper and washed with water. It was then transferred to 10 ml of 0.3 M sucrose, 0.1 M tricine–NaOH (pH 8.0) and homogenized at 0°C in a glass hand-held homogenizer. The homogenate was filtered through four layers of cheesecloth and assayed directly.

Enzyme assays

Phospholipase activity was routinely measured using the following new assay. The substrate, PC, was prepared by sonicating the desired combination of labelled PC (dipalmitoyl- l - 14 C) and unlabelled PC in 0.1% Triton X-100 in a bath sonicator (Bransonic 12, Branson Inc., Shelton, CT) until the mixed micelle emulsion became crystal clear. The standard reaction mixture (1 ml) contained 100 μ M PC-dipalmitoyl (50 000 ct min $^{-1}$), 0.01% Triton X-100, 100 mM glycine–NaOH (pH 9.0) and 50–400 μ l of culture filtrate, and was incubated in a shaking water bath at 30°C and 140 r min $^{-1}$. The reaction was simultaneously stopped and extracted by adding 50 μ l acetic acid, 7 ml 3:2 hexane/isopropanol (v/v) and 5 ml 6.7% Na $_2$ SO $_4$. The lipids were removed in the top phase and dried under a stream of N $_2$. Fifty micrograms each of oleic acid and PC (soybean) were added as carriers, and the lipid samples were spotted on 250 μ m Silica gel G TLC plates and developed in 70:30:1.5 hexane/diethyl ether/acetic acid (v/v/v). The free fatty acid and PC spots were visualized with I $_2$. The spots were scraped from the plates and radioactivity was quantitated with a Beckman scintillation counter using a fluor containing 0.4% PPO and 0.005% POPOP in toluene. The percent conversion of 14 C-PC to 14 C-free fatty acid was determined and used to calculate enzyme activity in the units of nanomoles of fatty acid released per hour per millilitre of culture filtrate. For verification of the apparent phospholipase B activity, PC, lyso PC and free fatty acids were separated by TLC developed in 85:15:10:3.5 chloroform/methanol/acetic acid/water (v/v/v/v).

Protein was measured by precipitation of the culture filtrates with 10% trichloroacetic acid (w/v), resuspension with 0.1 N NaOH, and assaying by the Lowry method [10]. Esterase activity was measured with ρ -nitrophenyl esters. The reaction

mixture contained 2 mM ρ -nitrophenylbutyrate, 0.1% Triton X-100, 50 mM K_2PO_4 (pH 8.0) and 50–200 μ l culture filtrate. The enzyme activity was monitored at 405 nm with a Beckman model 35 recording spectrophotometer.

When other compounds were tested as potential substrates, the reaction mixture (1 ml) contained 100 μ M substrate (except for cutin which was 5 mg), 0.01% Triton X-100, 100 mM glycine–NaOH buffer (pH 9.0) and 100–500 μ l culture filtrate. The reactions were stopped by extracting the lipids and analysing the liberated free fatty acids by the method of Nixon & Chan [15].

Cutin was purified as described by Baker & Bateman [1]. Monogalactosyldiacylglycerol and DGDG were purified from a spinach leaf extract by preparative TLC on 500 μ m Silica gel G plates, in 70:21:3 chloroform/methanol/water (v/v/v).

Joboba oil was obtained from the Office of Arid Land Studies, University of Arizona. Soybean phosphatidylcholine (Asolectin) was obtained from Associated Concentrates, Woodside, NY. ^{14}C -Phosphatidylcholine (100 Ci mol $^{-1}$) was obtained from New England Nuclear. All other reagents were obtained from Sigma.

RESULTS

Phospholipase activity in liquid cultures

Phytophthora infestans (race 4) grew well on liquid cultures of rye steep, V/8, pea, bean and Hohl's P-1 media. After 7 days of growth, low levels of phospholipase were detected in the culture filtrates of complete rye and V/8 media, but no activity was detected in filtrates of the other media (Table 1). The deletion of glucose from the rye medium resulted in a 15-fold stimulation in extracellular phospholipase activity. The subsequent addition of a low concentration of phospholipid (10 mg soybean PC/100 ml medium) to the modified rye medium caused an additional 35-fold stimulation in the activity of phospholipase. In contrast, the addition of phospholipid to the V/8 medium produced only a 10% stimulation of phospholipid activity. Even after the addition of phospholipid to pea, bean, and Hohl's P-1 media, no phospholipase activity was detected. This experiment and all others were performed at least three times, and the values reported are the average of at least three replicates.

Because phospholipase activity was apparently induced in the PC-amended rye medium (Table 1), we undertook a time-course study of phospholipase production in the various rye media (Fig. 1). Phospholipase activity was first detected on the fourth day after inoculation and the peak of activity occurred at day 7 in the rye plus PC medium. As in Table 1, phospholipase activity remained low in the complete rye medium (+glucose) and the modified rye medium (–glucose) during the entire 14-day period.

During the first 7 days, the presence of glucose had very little effect on the fresh (wet) weight of the fungus (Fig. 1). However, the addition of PC caused a 50% increase in fresh weight. This stimulation may be attributed to the metabolism of PC by the fungus. From days 7–14 there was no further growth in the rye, or rye and PC, but there was a doubling of the fresh weight with rye and glucose. The peak of phospholipase activity at day 7 corresponds to the end of the log phase and the beginning of stationary phase. Esterase activity (ρ -nitrophenyl butyrate hydrolase) was also stimulated by adding PC and was repressed by adding glucose (Fig. 1).

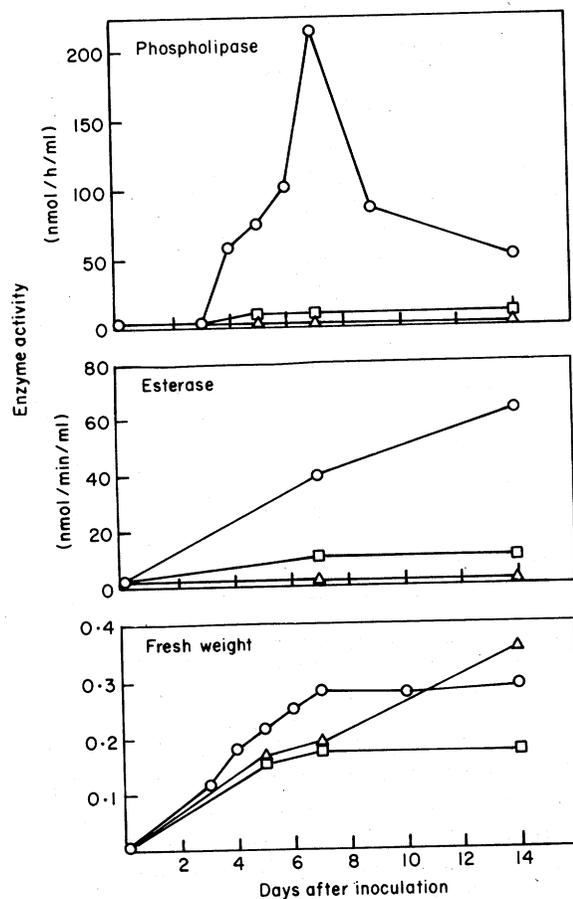


FIG. 1. Time-course study of the effect of the addition of PC (10 mg soybean) and glucose (1%) to modified rye medium (100 ml cultures). Enzyme activities were measured in the culture filtrates. Esterase activity was measured with p -nitrophenyl butyrate. For fresh (wet) weights mycelia were collected on filter paper with constant vacuum and weighed. Symbols: \square , rye; \circ , rye + PC; \triangle , rye + glucose.

However, the entirely different time courses of enzyme appearance suggest that the phospholipase activity (peak at day 7) and esterase activity (peak at day 14 or later) are two separate extracellular enzyme systems (Fig. 1). During growth (14 days) on the three media described in Fig. 1, the pH of the media increased from 5.8 to 7.0. The addition of buffer (50 mM potassium phosphate pH 6.0, 7.0, or 8.0) to rye + 10 mg PC reduced the production of phospholipase activity.

We then tested other compounds for their apparent ability to induce or repress phospholipase activity in rye medium (Table 2). A synthetic form of PC, dioleoyl, caused the highest stimulation of phospholipase activity, followed by PC from soybean (used in Fig. 1 and Table 1) triacylglycerol, Tween 20, PC (dipalmitoyl), wax ester, cholesterol oleate, autoclaved potato leaf and palmitic acid. The combination of PC

TABLE 2
Effect of the addition of various compounds to modified rye steep medium on the level of phospholipase activity in culture filtrates (7-day)

Compound	Rye steep medium (mg/100 ml)	Phospholipase activity (nmol/h ml)
None-control (-glucose)		6
PC (dioleoyl)	10	308
PC (soybean)	10	214
Triacylglycerol (sunflower)	20	171
Tween 20	50	95
PC (dipalmitoyl)	10	88
Wax ester (Jojoba oil)	20	86
PC (soybean) + Tween 20	10/50	63
Cholesterol oleate	20	58
Potato leaf (intact)	300	43
Palmitic acid	10	17
Oleoyl alcohol	20	5
Cutin (tomato fruit)	200	7
Tuber discs (potato)	1000	5
Glucose	1000	0.4
Glucose + PC (soybean)	1000/10	0.4
Glycerol	500	0.5

Extracellular phospholipase activity was measured in 7-day culture filtrates. All lipids were sonicated in 5 ml distilled water before addition to medium.

(soybean) and Tween 20 resulted in less stimulation of phospholipase activity than the single addition of either of these compounds. The addition of oleoyl alcohol, cutin or autoclaved potato tuber discs had no effect on the levels of extracellular phospholipase activity. Phospholipase activity was apparently repressed by the addition of glucose (as in Fig. 1 and Table 1) or glycerol. This apparent repression was not overcome by the addition of PC (soybean) in combination with glucose.

To determine the conditions for the optimal production of phospholipase activity, several concentrations of PC (dioleoyl) and PC (soybean) were added to rye medium (Fig. 2). The highest phospholipase activities were observed after the addition of 10 mg/100 ml of each of the two compounds. At 10 mg/100 ml, 44% more phospholipase activity was produced with PC (dioleoyl) than with PC (soybean). Because of its high cost, PC (dioleoyl) was not tested at higher concentrations. Phosphatidylcholine (soybean) at 10 mg/ml was subsequently used to induce phospholipase activity for the further enzymatic studies.

In addition to an extracellular phospholipase activity, *P. infastans* also has intracellular phospholipase activity (Table 3). However, the degree of stimulation of the intracellular activity by PC was much lower (10-fold) than that of the extracellular activity (41-fold). Because the peak of extracellular activity occurs at the end of the log phase (when most cells are still viable) (Fig. 1), it is most likely that the extracellular activity is actively secreted during growth and does not result from lysis of cells.

Phospholipase activity was then compared in the rye (+ 10 mg PC) culture filtrates of isolate R-4A (used for all previous studies) and three other isolates (Table 4). Phospholipase activity was only detected in R-4A and R-0A culture filtrates. These two

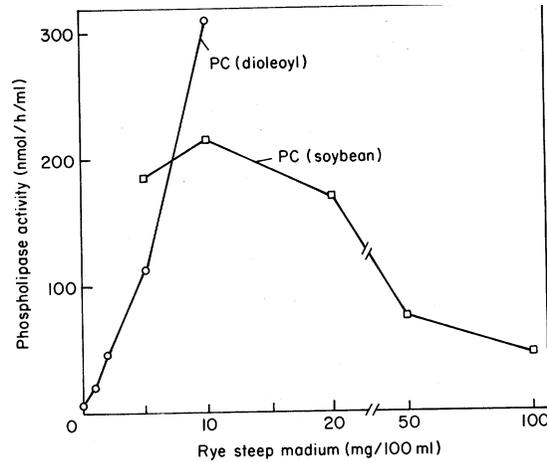


FIG. 2. Effect of the addition of various concentrations of PC to rye steep medium on phospholipase activity. Phospholipase activity was measured in the culture filtrates after 7 days of incubation.

TABLE 3
A comparison of intracellular and extracellular phospholipase activity in cultures of *P. infestans* (7-day cultures)

Medium	Total activity (nmol/h/culture)	
	Extracellular	Intracellular
Rye (- glucose)	525	85
Rye + 10 mg PC	21 750	877

TABLE 4
Phospholipase in culture filtrates of various isolates of *P. infestans* grown on liquid rye steep medium (+ 10 mg PC) for 7 days

Isolate	Physiologic race	Virulence on potato leaves	Phospholipase activity (nmol/h/ml)
R-4A	4	Low	247.6
R-0A	0	Low	187.4
R-0V	0	High	0
R-1,2V	1,2	High	0

isolates had been cultured on rye agar for several years and were essentially avirulent on potato leaves. In contrast, isolates R-0V and R-1, 2V, which were recently obtained and maintained on corn seed concoction to retain virulence [3], produced no detectable phospholipase activity in liquid rye (+ PC) cultures.

The ability of the PC-stimulated liquid rye culture filtrates to hydrolyse other acyl lipids was determined (Table 5). The synthetic esterase substrates (*p*-nitrophenyl

TABLE 5
Ability of the PC-stimulated liquid rye cultured filtrates to hydrolyse various acyl lipids

Lipid substrate	[s]	Hydrolytic activity (nmol/h/ml)
<i>p</i> -Nitrophenyl palmitate	0.2 mM	2334.0
<i>p</i> -Nitrophenyl butyrate	2.0 mM	2207.0
Triarachidonin	0.1 mM	55.88
Triolein	0.1 mM	52.19
1,3-Diolein	0.1 mM	52.60
1,2-Diolein	0.1 mM	52.07
Monoolein	0.1 mM	49.38
Lyso-PC (palmitoyl)	0.1 mM	45.94
PC (dipalmitoyl)	0.1 mM	44.69
DGDG	0.1 mM	38.28
MGDG	0.1 mM	36.88
Wax ester (stearyl stearate)	0.1 mM	<1
Cholesterol oleate	0.1 mM	<1
Cutin (tomato fruit)	5 mg/ml	<1

Enzyme activities were measured in 7-day culture filtrates of rye steep medium to which 10 mg of PC (soybean) had been added before inoculation.

butyrate and *p*-nitrophenyl palmitate) were hydrolysed at the highest rate. Because the data in Fig. 1 suggest that phospholipase and esterase activities are due to two different enzymes, we did not use *p*-nitrophenyl esters as model substrates for phospholipase activity. Among the lipid substrates, the various acylglycerols (mono, di and tri) were hydrolysed at the highest rates (Table 5), followed by lyso-PC, PC, MGDG and DGDG, and palmitic acid methyl ester. No detectable hydrolysis of tomato cutin, cholesterol oleate or wax ester was observed. Although the latter two apparently induced phospholipase activity (Table 2), the compounds themselves were not hydrolysed by the induced activity (Table 5). Table 5 suggests that PC induced either a broadly specific lipolytic acyl hydrolase enzyme [2] or separate phospholipase B, galactolipase (E.C.3.1.1.26), and acylglycerol lipase (E.C.3.1.1.3) enzymes.

The phospholipase activity was then further characterized. Phospholipase activity was nearly linear with 25–400 μ l culture filtrate (1–14 μ g protein), when incubated from 0–120 min. In order to determine whether this activity is catalysed by phospholipase A or phospholipase B, the substrate, PC (di-1-¹⁴C-palmitoyl), was separated from the potential products, lyso-PC and free fatty acids. Since no counts were detected in the lyso-PC spot (data not shown), the activity was apparently catalysed by a B-type phospholipase. This is consistent with the broad specificity shown in Table 5.

The extracellular enzyme(s) also had a low K_m (2.86 μ M) for PC (dipalmitoyl) (Fig. 3). When assayed at 25, 30 and 35 °C, the observed phospholipase activities were 34, 107 and 114 nmol/h/ml, respectively. The effect of various detergents on phospholipase activity was measured. The highest rates of hydrolysis were found with deoxycholate (0.1%), followed by Triton X-100 (0.01–0.05%), Tween 20 (0.1%), *n*-octylglucoside (30 mM) and, finally, Tween 80 (0.1%). Because deoxycholate interfered with the TLC analysis, 0.01% Triton X-100 was routinely used.

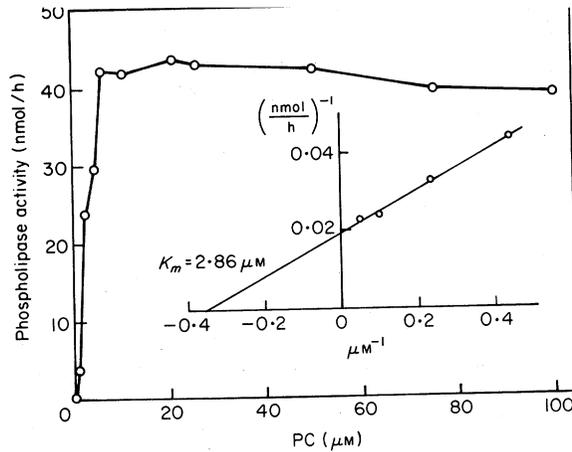


FIG. 3. Effect of concentration of substrate, PC (dipalmitoyl), on phospholipase activity. Culture filtrate from a 7-day PC-induced culture was used as a source of enzyme.

TABLE 6
Phospholipase in the wash of various isolates of *P. infestans* grown on bean agar media for 14 days

Isolate	Physiologic race	Phospholipase activity (nmol/h/ml)
R-0A	0	164.1
R-0V	0	703.9
R-1,2V	1, 2	794.0
R-4A	4	684.4

One petri dish culture was rinsed with 15 ml distilled water and the rinse was centrifuged and assayed for enzyme activity.

Phospholipase activity in agar cultures

An extracellular phospholipase was also detected in bean agar cultures of all four isolates of *P. infestans* (Table 6). Isolates R-0V, R-1,2V and R-4A all produced high levels of phospholipase activity. Isolate R-0A produced about fivefold less phospholipase activity than the other three isolates. Surprisingly, the two virulent isolates (R-0V, and R-1,2V) which produced no phospholipase activity in liquid culture (Table 4), were the highest producers of phospholipase activity on agar cultures (Table 5). These results suggest that the regulatory factors involved in the production of this enzyme activity are very different in liquid culture vs agar culture.

Phospholipase activity in infected potato leaves

Potato leaves were inoculated with zoospores of the R-1,2V isolate. The leaves were homogenized and assayed for phospholipase activity (pH 9.0) at 0, 3, 5, 7 and 14 days after inoculation (Fig. 4). Some phospholipase activity was present in uninoculated leaves as previously reported [13]. The activity increased about 30-fold during the first 5 days and then levelled off for the remainder of the experiment.

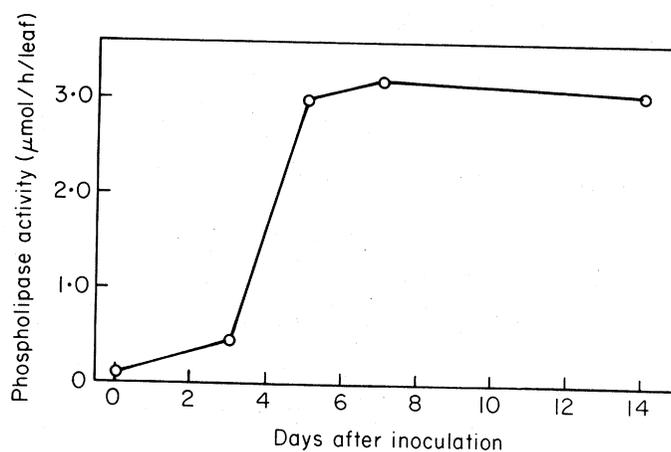


FIG. 4. Time-course study of phospholipase activity in potato leaves after inoculation with *P. infestans*. The reaction mixture (1 ml) consisted of 200 μl of 20 000 g supernatant of leaf homogenate, 100 mM glycine NaOH pH 9.0, 100 μM ¹⁴C-PC and 0.01% Triton X-100.

TABLE 7

Effect of various compounds on phospholipase activity from cultures of P. infestans and from uninfected and infected potato leaves at 7 days after inoculation

	Liquid rye culture ^a (100 ml) (nmol/h/ml)	Agar culture (15 ml) (nmol/h/ml)	Potato leaf (one leaf in 10 ml)	
			Infected (nmol/h/ml)	Uninfected (nmol/h/ml)
Control	109.0 (100.0)	124.8 (100)	315.8 (100.0)	22.9 (100)
5 mM DTT	4.0 (3.7)	5.8 (4.6)	31.1 (9.8)	19.1 (83.4)
5 mM EDTA	108.5 (99.5)	55.5 (44.4)	262.5 (83.1)	162.2 (70.8)
1 mg/ml BSA	101.0 (92.7)	149.7 (119.9)	189.2 (59.9)	3.82 (16.7)
5 mM MgCl ₂	72.5 (66.5)	—	—	—
5 mM CaCl ₂	103.5 (95.0)	103.70 (83.1)	—	—
5 mM β-mercaptoethanol	135.5 (124.3)	—	—	—

^aDialysed against 50 mM K₂PO₄ buffer (pH 7.0) for 18 h before use.

Each compound was mixed with the enzyme preparation and incubated for 5 min at 30°C before the reactions were started by addition of emulsified substrate.

Number in parenthesis is the relative activity (control = 100%).

Comparison of phospholipase activity from cultures and from infected leaves

Since phospholipase activity was detected in liquid cultures, agar cultures, uninfected and infected potato leaves, some of the properties of the enzymes from the four sources were compared.

Several potential inhibitors were tested for their effect on fungal and leaf phospholipases (Table 7). Dithiothreitol inhibited more than 90% of the phospholipase activity in both types of cultures and in infected leaves. The phospholipase activity in the uninfected leaves was only inhibited 17% by DTT, but it was severely inhibited (83%) by BSA. The other effects of BSA and EDTA were more variable

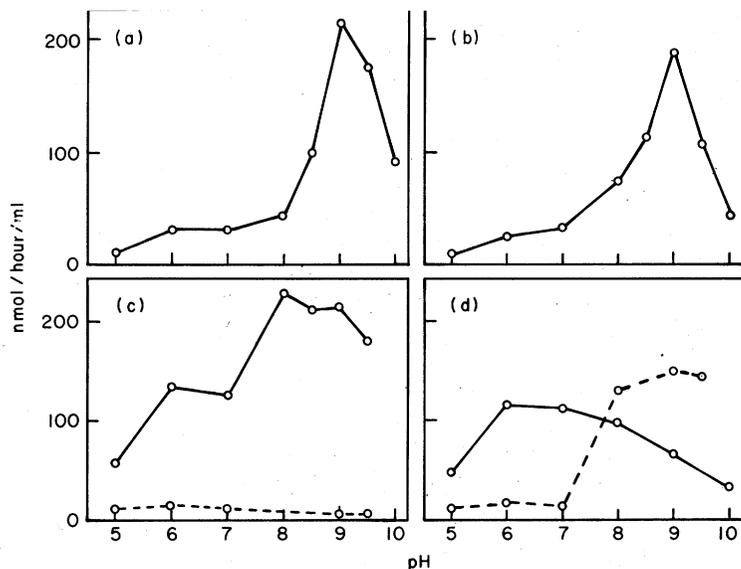


Fig. 5. Effect of pH on phospholipase activity in: (a) culture filtrates of isolate R-4A grown on rye steep media + 10 mg PC for 7 days; (b) culture wash of isolate R-1,2V grown on Lima bean agar for 14 days; (c) Katahdin potato leaf, uninfected (---) or infected (—) with isolate R-1,2V for 7 days; (d) infected Katahdin leaf (7 days) assayed in the presence of 5 mM DTT [DTT-insensitive (—) activity]. Dithiothreitol-sensitive activity (---) was calculated by subtracting the phospholipase activity in the presence of DTT from that measured in the absence of DTT.

and are probably a reflection of the variable concentrations of divalent cations and total protein in the enzyme preparations. Among the cultures tested, CaCl_2 and MgCl_2 were slightly inhibitory and β -mercaptoethanol increased the activity by 24%.

To further compare the phospholipase activities from the four sources, the effect of pH on enzyme activity was measured (Fig. 5). The activities from both types of fungal cultures exhibited maximal activity at pH 9.0 [Fig. 5(a), (b)]. The uninfected leaf had low, but detectable activity with a peak at pH 6.0 [Fig. 5(c)]. The infected leaf had two peaks of phospholipase activity [Fig. 5(c)], a large one with an optimal pH of 8.0–9.0, and a smaller peak at pH 6.0. These two peaks were further resolved [Fig. 5(d)] by assaying the phospholipase activity in the infected leaf in the presence of 5 mM DTT (DTT-insensitive activity). The DTT-sensitive activity in the infected leaf was then extrapolated by plotting the difference between phospholipase activity in the presence [Fig. 5(d)] and absence [Fig. 5(c)] of DTT. These results suggest that the DTT-sensitive alkaline phospholipase activity in infected leaves is of fungal origin. Further work is required to determine if the peak of DTT-insensitive activity is produced by the host or by the pathogen during infection.

DISCUSSION

It was previously shown that the growth of many species of *Phytophthora* was stimulated by phospholipids [7], triacylglycerols [5] and sterols [5, 7]. With two out of five isolates

of *P. infestans* there was a stimulation (10–20%) in growth after addition of phospholipid to the medium [7]. In Fig. 1 it can be seen that the inclusion of glucose to the rye medium had very little effect on growth of *P. infestans* before 7 days, while the addition of PC stimulated growth by about 50%.

This is the first report of the optimal conditions for production of an extracellular phospholipase from a phytopathogen. In the two previous phospholipase studies that reported the addition of PC to the medium to induce phospholipase activity, the effect of PC on the activity of phospholipase was not presented [17, 20]. A very dramatic degree of stimulation of phospholipase activity (770-fold) was observed under optimal conditions, +PC (dioleoyl) in Table 2, as compared with the complete (+ glucose) rye medium.

Our results suggest that this large stimulation was probably caused by the induction of phospholipase activity by PC, and repression by glucose. However, the complex natural media which we were forced to use (Table 1), and the possibility that we were measuring the combined activity of more than one enzyme species (Table 5), make it impossible to verify this hypothesis.

The ability of an extracellular culture filtrate to hydrolyse lipids other than phospholipids (Table 5) has not been reported previously for any other phytopathogen. Since galactolipids are actually the most abundant lipids in the leaves of most species [4], an enzyme which is capable of degrading them may be as important as a phospholipase during pathogenesis. During the infection of bean leaves by *U. phaseoli*, galactolipids are degraded to a greater degree than phospholipids [8].

Several enzymatic properties of this phospholipase are unique. The extracellular B-type phospholipases from *S. sclerotium* [12], *S. rolfsii* [19], *B. cinerea* [17] and *T. basicola* [11] all had acid pH optima. *Thielaviopsis basicola* had a second peak of phospholipase activity at pH 8.5; but this activity was stimulated by Ca^{++} [11]. Although the extracellular phospholipase activity of *P. infestans* also had an alkaline pH optimum (Fig. 4), it was not stimulated by Ca^{++} or Mg^{++} (Table 7). The stimulation of phospholipase activity by β -mercaptoethanol, and the almost complete inhibition by DTT is an unusual observation which we intend to study in more detail with purified enzymes.

The four isolates of *P. infestans* that were used in this study produced different amounts of phospholipase activity in liquid and in agar cultures. The more virulent isolates produced no phospholipase activity in liquid cultures, but produced the highest levels of phospholipase in agar cultures. *Phytophthora infestans* is known to lose virulence after several months of growth on most types of culture media [3, 6]. Very little is known about the physiological changes that occur during loss of virulence. Further study is required to determine whether there is a true correlation between phospholipase production in liquid culture and avirulence. Interestingly, there was also no correlation between physiologic race and phospholipase production in liquid or agar cultures.

Our studies with infected leaves suggested that *P. infestans* does produce the same DTT-sensitive alkaline phospholipase activity in infected leaves as we observed in culture. The second peak of phospholipase activity in infected leaves may also be of fungal origin, or it may be caused by increased production of the leaf enzyme (which also has a pH optima at 6.0) during infection.

Reference to brand or firm name does not constitute endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

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