AN EXTRACELLULAR PHOSPHATIDASE PRODUCED BY *BOTRYTIS CINEREA IN VITRO*[1]

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Abstract

An extracellular phosphatidase produced by *B. cinerea* grown on bean hypocotyl medium was demonstrated. This enzyme exhibited a maximum activity at pH 5.0. It was stable after exposed to 35 C for 30 min at pH 5.0; but the activity was completely lost after 40 C treatment for 30 min. The enzyme was inhibited by 10 μ M sodium cyanide. However there was no calcium ion requirement for the enzyme activity. The phosphatidase was able to release palmitic acid, linoleic acid and glycerylphosphorylcholine from soybean lecithin. These evidences indicated that the enzyme produced by *B. cinerea* was phosphatidase B.

Introduction

The involvement of enzymes of pathogen origin in pathological processes is an accepted fact. Our knowledge of pectic enzymes in plant pathogenesis is perhaps more complete than that for any other group of enzymes (Bateman and Millar 1966; Husain and Kelman 1959; Wood 1960). Brown (1915) observed that culture filtrates of *Botrytis cinerea* readily macerated plant tissues and that tissue maceration were accompanied by cellular death. Attempts to separate the factors responsible for tissue maceration and death of plant cells in culture filtrates of pathogens have so far been unsuccessful (Brown 1915; Lai *et al.* 1968; Thatcher 1942; Tribe 1955).

The loss in the semipermeability of cell membranes is associated with cellular death. There is evidence that these permeability changes can be attributed to enzymes of pathogen origin in diseases involving such pathogens as *Rhizoctonia soloni* (Lai *et al.* 1968), *Botrytis cinerea* and *Sclerotinia sclero-*

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tiorum (Thatcher 1942). The type of enzymes responsible for this alternation of suscept membranes has not been elucidated, but lecithinases (phosphatidases) have been suggested as likely enzymes to effect such permeability changes (Thatcher 1942; Tribe 1955).

In the current survey of the ability of a number of phytopathogens to produce phosphatidases *in vitro*, *B. cinerea* was one of the most active in this capacity of the tested organisms (Tseng and Bateman 1968). The investigation reported here was undertaken to determine the properties of the phosphatidase produced by *B. cinerea* and to determine the type of phosphatidase produced by the fungus.

Materials and Methods

B. cinerea Person was used throughout this investigation. The cultures were maintained on potato-dextrose agar (PDA) at 25 C and transfers were made at one month intervals. Production of phosphatidase was made by growing *B. cinerea* on bean (*Phaseolus vulgaris* var. Red Kidney) hypocotyl medium. Healthy bean hypocotyls were obtained from plants growing in steamed soil in a growth chamber at 29–30 C for 7 days. They were stored at –20 C until needed. The medium for phosphatidase production was prepared by autoclaving 50 g of bean hypocotyls with 20 ml of distilled water in 1 liter Erlenmeyer flasks for 30 min at 121 C. The sterilized medium was seeded with one-week-old culture of the organism and incubated at 28±2 C for 10 days.

Culture grown on the bean hypocotyl medium was harvested by grinding the flask contents with 50 ml of distilled water in a Virtis '45' homogenizer for one minute at high speed. The extracts were filtrated through 4 layers of cheesecloth to remove solid materials and then centrifuged at 20,000 *g* for 15 min at 5 C. The supernatants were lyophilized and stored at –20 C in a dissicator until used.

Enzyme assays.—Refined soybean lecithin (Nutritional Biochemicals Corp., Cleveland, Ohio) was used as the substrate in all enzyme assays. One per cent aqueous emulsion of lecithin was prepared by blending 1.0 g of the soybean lecithin with 100 ml of buffer (0.1 M) containing 0.01% Thiomersalate (Eli Lilly Co.) in a Virtis '45' homogenizer at full speed for 30 min. The homogenizer vessel was kept in an ice bath to prevent overheating. The lecithin emulsion was kept in a refrigerator at 4 C until used. Lyophilized culture extracts were used as enzyme sources.

Phosphatidase activity was estimated by measuring acyl-ester contents of the substrate in the reaction mixture using the procedures described by Snyder and Stephens (1959). Reaction mixtures consisted of 0.5 ml lecithin (2.7 μ moles) in 0.1 M buffer (citrate or phosphate) and 0.5 ml of enzyme preparations. The

mixtures were incubated for the stated period at 30 C in centrifuge tubes. The reaction mixture was stopped by adding 0.1 ml 5% (w/v) bovine serum albumin (BSA) to aid precipitation of phosphatides, and 0.9 ml 20% trichloroacetic acid (TCA). The contents of tubes were then centrifuged at 20,000 g for 15 min at 5 C. After centrifugation, the supernatants were discarded, 2.0 ml of alkaline hydroxylamine reagent were added to the firm pellets remaining in centrifuge tubes. The samples were placed in a 65 C water bath for 3 min, with shaking, removed and allowed to cool for 5 min at room temperature. Five ml of ferric perchlorate reagent were added and the samples were

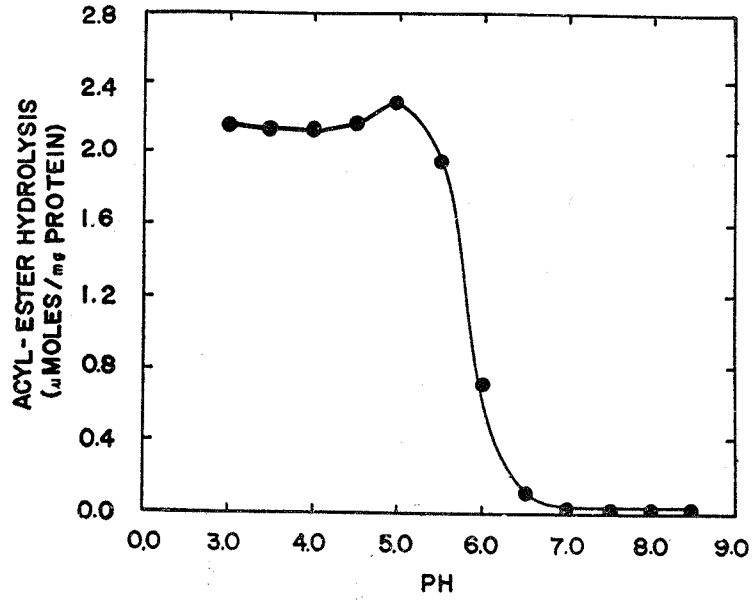

Fig. 1 Effect of pH on the activity of phosphatidase from *B. cinerea* grown on bean hypocotyl medium. Reaction mixtures contained 0.5 ml enzyme (0.48 mg protein) and 0.5 ml lecithin (2.75 μ moles) in 0.1 M buffer. Two buffer systems were used for this purpose. Citrate buffer (0.1 M) for pH 3.0-6.5; phosphate (0.1 M) buffer for pH 7.0-8.5. The reaction was carried out at 30 C for 24 hr.

centrifuged. Thirty minutes after, the supernatants were read against a reagent blank at 530 mμ in a "Spectronic 20", and the amount of acyl-ester contents in reaction mixture was calculated. Acyl-ester contents were estimated by using a standard curve prepared with known amounts of lecithin. One unit of enzyme activity was defined as the amount of enzyme hydrolysis 1.0 μ equivalent of acyl-ester bond in reaction mixture at 30 C for 1 hr (Fig. 2). Protein meaurements were made by using the method of Lowry *et al.* (1951).

Identification of the reaction products of the hydrolysis of lecithin by ***B. cinerea*** *phosphatidase.*—The hydrolysis of fatty acids from lecithin by the phosphatidase

produced by *B. cinerea* was determined by gas liquid chromatography, and by following the decrease in the acyl-ester contents of reaction mixture. Reaction mixtures contained 0.5 ml of enzyme (0.56 mg protein) and 0.5 ml of 2.75 μ moles of lecithin in 0.1 M buffer (citrate buffer for pH 3.0–6.5; phosphate buffer for pH 7.0–8.5). The mixture was carried out at 30 C for 2 hr.

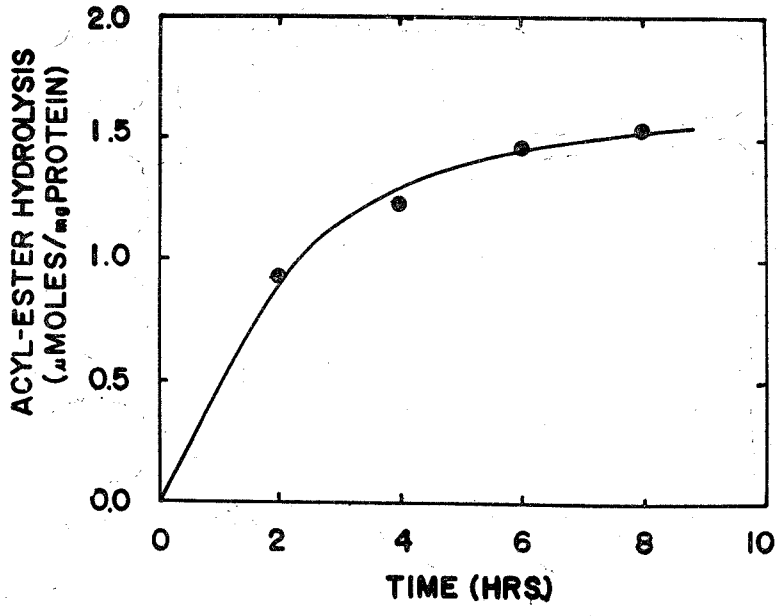

Fig. 2 The rate of acyl-ester hydrolysis in soybean lecithin by the phosphatidase from *B. cinerea* grown on bean hypocotyl medium. Reaction mixture contained 0.5 ml enzyme (0.56 mg protein) and 0.5 ml lecithin (2.75 μ moles) in 0.1 M citrate buffer at pH 5.0.

Reaction mixtures were extracted for free fatty acids as previously described (Tseng and Bateman 1969). The fatty acids were methylated by using methanolic BF3 (14% w/v-Applied Science Laboratories, State College, Pa.). These samples were then assayed for fatty acid methyl esters by gas liquid chromatography (GLC). Assays on the samples were made by using an Aerograph (Model 1200) Gas Chromatograph equipped with $^1/_8$ inch O. D. 5-ft column packed with 3.0% General Electric SE-30 silicone gum on 100–120 mesh Diatoport S. and with a hydrogen flame detector. During analysis injector, column, and detector temperatures were maintained at 215, 195, and 250 C, respectively. Nitrogen was used as the carrier gas and a flow rate at 25 ml/min.

For determination the acyl-ester contents in the reaction mixtures, the procedures were exactly the same as described above. After centrifugation,

the firm pellets in the centrifuge tubes were used to determine the amount of remaining acyl-ester bonds in the reaction mixtures, and the supernatants were assayed for glycerylphosphorylcholine (GPC) by the procedure described by Barron (1964), and for free choline by the method of Appleton *et al.* (1953).

Results.

It was demonstrated that the culture extract of *B. cinerea* contained active phosphatidase. Assay for healthy bean hypocotyls did not contain any detectable phosphatidase activity even after 24 hr incubation.

***B. cinerea** Phosphatidase activity in relation to pH.*—The influence of pH on the hydrolysis of lecithin by the enzyme preparation was shown in Fig. 1. Two buffer systems were used for the wide span of pH values tested. Citrate buffer (0.1 M) was used for pH 3.0–6.5; phosphate buffer (0.1 M) was served for pH 7.0–8.5. Acyl-ester hydrolysis of lecithin was maximal at pH 5.0, the pH optima were distinctly in the acid range, no enzyme activity was detected over the pH 7.0.

Stability of the phosphatidase to heat.—The stability of the phosphatidase from *B. cinerea* was examined by incubating 15 ml samples containing 1.12 mg/ml protein that had been dialyzed overnight against several liters of distilled water in a water bath at 35 C and 10–70 C at 10 C intervals for 30 min at pH 5.0. After the temperature treatments, assay was made by acyl-ester method and the enzyme incubated at 0 C served as the control. Fig. 3 showed that there was no loss in phosphatidase activity in the 10, 20, 30, and 35 C treatments as compared to the control. However, the enzyme was completely inactivated at 40 C.

Inhibition of the phosphatidase activity by sodium cyanide.—The effect of sodium cyanide at concentrations of 10^{-5} to 10 μ moles/ml of reaction mixture was tested upon the phosphatidase produced by *B. cinerea*. Fig. 4 showed the results of sodium cyanide effect on the phosphatidase activities. It indicated that the enzyme was completely inhibited by 10 μ moles of sodium cyanide.

Effect of salts and EDTA on phosphatidase activities.—The effect of chloride salts of Ca^{++}, Mg^{++}, $Cu,^{++}$ and $K,^{+}$ as well as EDTA at concentration of 0.125, 1.25, and 12.5 mM/ml of reaction mixtures was tested upon the phosphatidase activities at pH 5.0. Enzyme preparation for this purpose was dialyzed against several liters of distilled water at cold room for 30 hr. The phosphatidase activity was not appreciably influenced by any additives as compared to the control, although a certain phosphatidases were known to be stimulated by Ca^{++} ions.

Identification of the phosphatidase produced by B. cinerea.—The free fatty acids released from refined soybean lecithin after a 2 hr incubation with

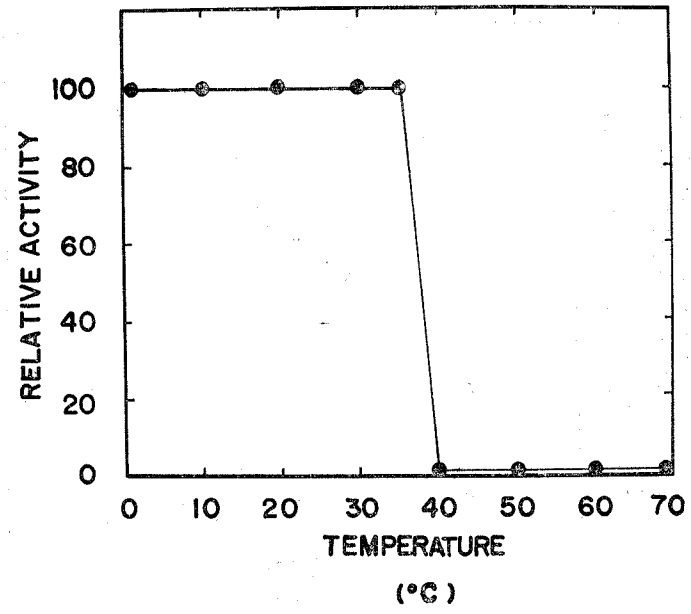

Fig. 3 The stability of phosphatidase from *B. cinerea* grown on bean hypocotyl medium. The enzymes were treated with the indicated temperatures for 30 min. Reaction mixture contained 0.5 ml of treated enzyme (0.56 mg protein) and 0.5 ml lecithin (2.75 μ moles) in 0.1 M citrate buffer at pH 5.0. The mixtures were incubated at 30 C for 6 hr. Enzyme incubateed at 0 C served as control. Relative activity represents the activity in treatments relative to that of the treatment exhibiting maximum activity in the given experiment as determined by the acyl-ester assay.

phosphatidase obtained from *B. cinerea* culture extracts revealed that palmitic and linoleic acids were released only in the presence of active enzyme (pH 5.0) (Fig. 5). These fatty acids were identified by their retention times on GLC column (SE-30), and by cochromatography with known fatty acid methyl esters. Since pamitic and linoleic acids represent the major fatty acid constituents in Soybean lecithin, these fatty acids released in the reaction mixture indicated that *B. cinerea* phosphatidase was capable of releasing the fatty acids from both the α and β positions of lecithin molecule.

The release of the fatty acids from lecithin was confirmed by determing the decrease in acyl-ester contents of reaction mixtures when incubated with *B. cinerea* phosphatidase. The nonhydrolyzed lecithin in 1.0 ml reaction mixture consisting of 0.5 ml enzyme (0.56 mg protein) and 0.5 ml lecithin (2.75 μ moles) in 0.1 M citrate buffer (pH 5.0) was precipitated by the addition of 0.15 ml 5% BSA and 0.5 ml 20% TCA. The precipitate was recovered by centrifugation, and its acyl-ester content was determined. The supernatants from the

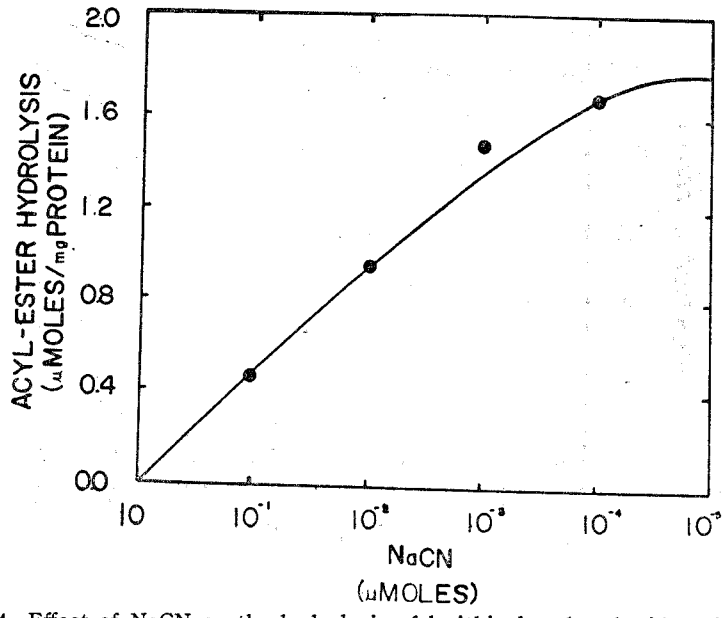

Fig. 4 Effect of NaCN on the hydrolysis of lecithin by phosphatidase from *B. cinerea* grown on bean hypocotyl medium. Reactin mixture contained 0.5 ml enzyme (0.56 mg protein) and 0.5 ml lecithin (2.75 μ moles) in 0.1 M citrate buffer at pH 5.0, the mixtures were incubated at 30 C for 18 hr.

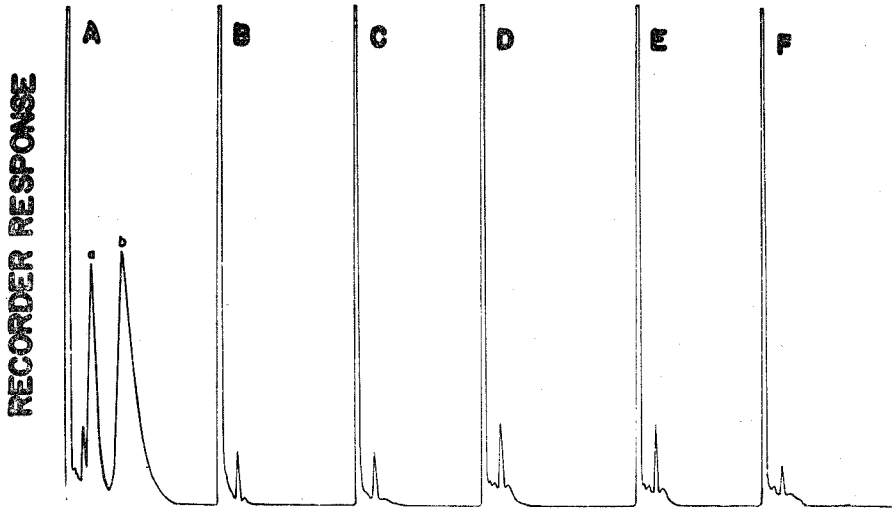

Fig. 5 Results of a gas chromatographic assay for the release of fatty acids by the phosphatidase produced by *B. cinerea* grown on bean hypocotyl medium. Reaction mixtures were carried out at 30 C for 2 hr.

A) Active enzyme at pH 5.0 B) Actoclaved enzyme at pH 5.0
C) Active enzyme at pH 7.0 D) Autoclaved enzyme at pH 7.0
E) Active enzyme at pH 8.0 F) Autoclaved enzyme at pH 8.0

The fatty acid released at pH 5.0 were identified as a) palmitic acid and b) linoleic acid.

reaction mixtures were assayed for TCA soluble products released from lecithin. Those results were shown in the Table 1.

Table 1. *Specificity of B. cinerea phosphatidase action a)*

Treatment	Decrese in acyl ester content (μ moles)	Increase (μ moles) of	
		Glycerylphosphoryl-choline	Free choline
Hydrolysis b)	0.86	0.84	0
Non-hydrolysis c)	0.85	0	0

a) Reaction mixture contained 0.5 ml of enzyme (0.56 mg protein) and 0.5 ml of lecithin (2.75 μ moles) in 0.1 M citrate buffer at pH 5.0. Incubation was carried out at 30 C for 2 hr.

b) At the end of 2 hr incubation, 0.5 ml of 5% bovine serium albumin (BSA) was added to the reaction mixture and the protein precipitated with 0.5 ml of 10% trichloroacetic acid and removed by centrifugation. Then 0.25 ml of 5 N HCl was added to 1.1 ml of the supernatant and the sample hydrolyzed for 20 min at 100 C*. The hydrolysate was centrifuged and the supernatant was analyzed for choline by the method of Appleton *et al.* (1953). The increase in choline liberated by acid hydrolysis of glycerylphosphorylcholine expresses the amount of GPC in the supernatant.

c) The reaction mixture was not hydrolyzed by 5 N HCl.

* During the hydrolysis, choline was liberated from glycerylphosphorylcholine while phosphorylcholine was not hydrolyzed under these conditions.

No evidence was obtained for the release of free choline or phosphorylcholine, supernatants obtained from reaction mixture containing active enzyme contain glycerylophosphorylcholine (GPC). Furthermore, the decrease in acyl-ester content was nearly equivalent to the increase in choline liberated by acid hydrolysis (glycerylphosphorylcholine). These results revealed that the phosphatidase produced by *B. cinerea* was phosphatidase B.

Discussion.

The ability to produce phosphatidases is widely distributed among phytopathogens (Mount and Bateman 1969; Pellezzari *et al.* 1970; Tseng and Bateman 1968). *B. cinerea* which is normally associated with soft rot of plant tissues produced considerable amount of phosphatidase B in the culture extracts. The characteristics of enzyme is similar to those of the phosphatidase obtained from culture filtrates of *Sclerotium rolfsii* as well as from extracts of infected bean hypocotyls (Tseng and Bateman 1969).

Enzyme with phosphatidase B activity is known to occur in certain mold and animal tissues with the maximun activity at pH 3.5–4.4 and at the pH 6.0–6.5, respectively (Kates 1960). The enzyme detected from *B. cinerea* growing on bean hypocotyl medium, exhibited maximun activity at pH 5.0. It was stable when exposed to 35 C at pH 5.0 for 30 min, and the enzyme

required no calcium ion for its activity. In contrast, the phosphatidase B from snake venom and that of bee are extremly stable to heat, both are activated by Ca^{++} and Mg^{++} ions (Doery and Pearson 1964). Apparently the phosphatidase systems produced by different pathogens and sources differ considerably from each other.

The phosphatidases produced by animal pathogens, snakes and certain insects have been implicated as toxicological agents in pathogenesis. Condrea and DeVries (1965) have reviewed the action of venom phosphatidase A on cellular membranes, they found that the hemolysis of red blood cells treated with snake venom had been attributed to phosphatidase A action on the cell membrane. The mitochondria in intact and isolated skeletal muscle had been observed to be disrupted when the tissue was treated with the alpha toxin of *Clostridium welchii;* this disruption of mitochondria was attributed to the hydrolysis of the mitochondrial lipids by the toxin (Slein and Logan 1965). However, the toxicity of the phosphatidases in plant tissues has not been elucidated.

B. cinerea is a good source for obtaining phosphatidase B. Recent report by Tseng and Lee (1969) revealed that the fungus produced a certain amount of proteolytic enzymes in culture filtrates. Since phosphatides and protein are the major constituents of plant membrane systems (Butt and Beevers 1966), the further investigation of the mechanisms of these enzymes in plant pathogenesis may provide a better understanding of disease processes.

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