Bot. Bull. Academia Sinica **17**: 111-125 (1976)

PURIFICATION OF ENZYMES (PHOSPHATIDASE C, PROTEASE, AND *ENDO*-POLYGALACTURONATE *TRANS*-ELIMINASE) PRODUCED BY *ERWINIA CAROTOVORA* AND THEIR EFFECTS ON THE ACTIVITY OF POTATO MITOCHONDRIA[1,2]

TSUNG-CHE TSENG

Institute of Botany, Academia Sinica, Nankang, Taipei
Taiwan, Republic of China

(Accepted for publication February 26, 1976)

Abstract

Three extracellular enzymes, *endo*-polygalacturonate *trans*-eliminase, phosphatidase C and protease, produced by *Erwinia carotovora* (isolate 14) were purified by ammonium sulfate fractionation, DEAE cellulose column chromatography, and isoelectric focusing. The crude enzymes were purified to a 23-fold for phosphatidase C, a 57-fold for protease, and a 141-fold of *endo*-polygalacturonate *trans*-eliminase. The isoeleotric points of the enzymes were: 7.5, 8.3, and 9.5 for phosphatidase C, protease, and *endo*-polygalacturonate *trans*-eliminase, respectively.

Purified phosphatidase C and protease affected the respiratory mechanism of mitochondria resulted in decrease in oxygen uptake. The inhibitory effect of phosphatidase C on mitochondria was much greater than that of protease, whereas purified *endo*-polygalacturonate *trans*-eliminase exhitited no appreciable effect. In contrast, *endo*-polygalacturonate *trans*-eliminase caused a significant decrease in respiration of potato discs, while phosphatidase C and protease showed only a slight effect.

Introduction

Bacterial soft rot of plant tissues is of world-wide cocurrence. It is one of the important diseases of vegetables and ornamental plants in transit and storage, and is among the most important of the bacterial diseases of growing plants. The destructive soft rots are usually caused by bacteria in the genus *Erwinia*, of these, *Erwinia carotovora* is the most prevalent.

Soft rots caused by bacteria usually develop rapidly in parenchyma and

(1) This work was partly supported by the National Science Council, Republic of China.
(2) Paper No. 174 of the Scientific Journal Series, Institute of Botany, Academia Sinica.

chlorenchyma of infected plants. Symptoms are finally death of protoplasts. The middle lamella is first attacked by enzymes which produced by the pathogens. Pectic compounds in the middle lamella and polygalacturonide-rich compounds in the matrix of the cell wall are hydrolyzed, resulting in loss of coherrence (maceration) of tissue. The degradation of pectic substances is accomplished by a variety of enzymes. Turner and Bateman (1968) showed that the macerating enzyme produced by *E. carotovora* was *endo*-polygalacturonate *trans*-eliminase (*endo*-PGTE), and tissue maceration was closely correlated with *endo*-PGTE activity. The events of maceration and cellular death of infected plants parallel each other (Garebaldi and Bateman, 1971). Attempts to separate maceration and cellular death as a result of pectic enzyme action have failed. Mount *et al.* (1970) demonstrated that a highly purified *endo*-PGTE produced by *E. carotovora* induced electrolyte loss, tissue maceration, and cellular death of potato tubers. They suggested that possibly a substrate for *endo*-PGTE residued in the plant cell membrane or within the protoplast. However, further study revealed both purified phosphotidase C and protease caused cucumber protoplasts to burst, whereas *endo*-PGTE did not. Thus the results indicated that *endo*-PGTE had no apparent effect on the membrane of cucumber protoplasts, and that phosphatidase and protease of *E. carotovora* played a role in disease development (Tseng and Mount, 1974).

Studies relating to organelle injury (such as mitochondria) by highly purified extracellular enzymes produced by soft rot pathogens are meager, except *Sclerotium rolfsii* phosphatidase B that inhibited mung bean mitochondrial oxygen uptake (Tseng and Chang, 1974). To understand the true enzymatic nature of host injury, particularly in organella level, induced by a pathogen is an essential step towards understanding pathogenesis. This paper presents the purification of phosphatidase C, protease, and *endo*-PGTE produced by *E. carotovora* and the effects of these enzymes on potato mitochondria in relation to respiratory activity.

Materials and Methods

Erwinia carotovora (Jones) Holland, isolate 14, was used throughout this investigation. This isolate is highly virulent on potato (*Solanum tuberisum* L.) and bean (*Phaseolus vulgaris* L.). It was maintained on nutrient agar slant at 25°C. Stock cultures were transferred at 2-week intervals.

The medium for phosphatidase, protease, and *endo*-PGTE production was bean (*P. vulgaris* var. Red Kidney) hypocotyl medium. Healthy bean hypocotyls were obtained from plants grown in greenhouse at 29–30°C for two weeks. It was prepared by autoclaving 60 gm of bean hypocotyls with 20 ml of distilled water in 1 liter Erlenmeyer flask for 30 min at 121°C. The sterile medium

was spreaded with a week-old culture and incubated at 28°C for 2 weeks. The crued cell-free extract of rotted tissue was obtained by grinding the flask contents with 50 ml of distilled water in a Virtis '45' homogenizer for one minute at high speed. The extract was filtrated through 4 layers of cheesecloth and then centrifuged at 20,000 g for 15 min at 5°C. It was then dialyzed against distilled water and lyophilized, and stored in dessicator at −20°C until used.

All of the chemicals used for the study were purchased from Sigma Chemical Company (St. Louis, Mo. 63178, U. S. A.).

Enzyme assay

Endo-polygalacturonate *trans*-eliminase (*endo*-PGTE)—The enzyme activity was measured by periodate-thiobartituric acid method (Preiss and Ashwell, 1963). The reaction mixture contained 0.1 ml of 0.6% (w/v) sodium polypectate in 0.05 M Tris (hydroxymethyl) amino methane (Tris)-HCl buffer (pH 8.0) plus 10^{-4} M $CaCl_2$ and 0.1 ml enzyme. After incubation for 1 hr at 30°C, 0.3 ml of 0.05 N $NaIO_4$ in 0.25 N H_2SO_4 was added to the reaction mixture and held at room temperature for 2 min. This was followed by the addition of 0.5 ml of 2% (w/v) sodium arsenite in 0.5 N HCl and the mixture was allowed to stand at room temperature for 2 min. Two mililiters of 30% (w/v) thiobartituric acid (pH 2.0) was then added and the mixture was placed in a boiling water bath for 10 min. Activity of the enzyme was detected by the formation after heating of a red chromogen which shows a maximum absorption at 548 nm. One unit of *endo*-PGTE was defined as that amount of enzyme giving an increase in absorbance of 0.1 in 1 hr at 548 nm.

Protease—The assay was performed by using spectrophotometric techniques (Kunitz, 1974). The reaction mixture consisted of 1.0 ml of 1.0% (w/v) gelatin (Nutritional Biochemical Corp.) in 0.05 M Tris (hydroxymethyl) amino methane (Tris)-HCl buffer (pH 8.0) with 0.01% $CaCl_2$ and 1.0 ml of enzyme preparation. Incubation was carried out at 37°C for 1 hr. At the end of the incubation period, 3.0 ml of 20% trichloroacetic acid (TCA) was added and the contents were thoroughly mixed. They were centrifuged at 5,000 g for 20 min at room temperature. The absorbance of the clear supernatant, containing TCA soluble products, was determined at 280 nm in a Beckman DU Spectrophotometer against a reaction blank. The reaction blank was identical to the reaction mixture except that TCA was added immediately following the addition of 1.0 ml of enzyme preparation. One unit of protease activity was defined as that amount of enzyme causing an increase in absorbance of 0.01 at 280 nm in the TCA soluble reaction products.

Phosphatidase—Since the phosphatidase produced by *E. carotovora* has been identified as a phosphatidase C (Mount and Bateman, 1969), the enzyme activity

was assayed by measuring the release of acid-soluble phosphorus (phosphorylcholine) from soybean lecithin as described by Lumsden and Bateman (1968). The reaction mixture contained 1.0 ml of 0.1% refined soybean lecithin emulsion in 0.05 M Tris-HCl buffer (pH 8.0), 0.5 ml enzyme, and 0.5 ml of 0.05 M Tris-HCl buffer (pH 8.0) with 0.25 M $CaCl_2$. The reaction mixtrue was incubated for 1 hr at 30°C and enzyme activity was stopped by the addition of 0.1 ml of 20% trichloroacetic acid. They were centrifuged at 28,700 g for 20 min at 4°C. One milliliter of the clear supernatant fluid was transferred to a test tube cover with a glass ball and was evaporated to dryness in a boiling water bath.

One tenth milliliter of concentrated nitric acid and 0.9 ml of 70% perchloric acid were added to each tube, and the organic contents were digested for 30 min at 170°C. After cooling the digest at room temperature, 7.75 ml of distilled water was added. The mixture was vigorously stirred, and 1.0 ml of a 2.5% aqueous solution of ammonium molybdate was added to each tube, immediately followed by the addition of 0.25 ml of 0.5 g 1-amino-2-naphthol-4-sulfonic acid dissolved in 195 ml of 15% (w/v) sodium bisulfite plus 5 ml of 20% (w/v) sodium sulfite. The mixture was shaken thoroughly and, after exactly 10 min, the absorbance at 650 nm was read in a B & L Spectronic 20 Colorimeter. The phosphorus concentration was determined by using a standard curve prepared with KH_2PO_4. One unit of enzyme activity was defined as the amount of enzyme releasing 10 μg of acid-soluble phosphorus in 1 hr at 30°C.

All of the controls except the one for the protease assay contained autoclaved enzymes instead of active enzymes using the spectrophotometric method. Protein was determined by the method of Lowry *et al.* (1951) using crystalline bovine albumin as standard.

Procedures of the enzyme purification

Step 1. Fractionation of enzyme preparations—Lyophilized culture extracts of *E. carotovora* (grown on bean hypocotyls) served as the crude enzyme source. Seven grams of lyphilized extracts was dissolved in 10 ml of cold distilled water. This procedure was repeated for ammonium sulfate saturation of 60, 80, and 90%. The precipitate of each fraction was immediately assayed for protein content and activity of phosphatidase C, protease, and *endo*-polygalacturonate *trans*-eliminase. Each fraction was dialyzed and separately subjected to diethylaminoethyl cellulose (DEAE cellulose, Cl form) column chromatography.

Step 2. Ion-exchange chromatography (DEAE cellulose column)—Partially purified enzymes (phosphatidase C, protease, and *endo*-polygalacturonate *trans*-eliminase from ammonium sulfate fractions) were applied onto a 2.5×23 cm of DEAE cellulose column buffered with 0.05 M Tris-HCl buffer (pH 8.0) at 5°C.

The column was eluted with 60 ml of 0.05 M Tris-HCl buffer (pH 8.0) followed by a NaCl stepwise gradient in buffer until 0.4 M NaCl was reached. Ten-ml fractions were collected and assayed for enzyme activities.

Step 3. Isoelectric focusing—The peak activity fractions of each of the three enzymes (phosphatidase C, protease, and *endo*-polygalacturonate *trans*-eliminase), eluted from the DEAE cellulose column were combined and dialyzed for 24 hr at 4°C. These enzymes were subjected to isoelectric focusing in a LKB 8101 Ampholine electrofocusing apparatus equipped with a 110-ml column (LKB Produkter AB, Bromma, Sweden) containing pH 7–10 Ampholine carriers. Electrofocusing was carried out at 4°C for 48 hr, at that time a stable pH gradient had formed. Five ml fractions were collected and the pH value of each fraction was measured. Each fraction was dialyzed against distilled water at 4°C for 24 hr and then assayed for protein content and enzyme activities. This procedure was used to determine the isoelectric point of the enzyme as well as the final step in its purification.

Isolation of mitochondria from potato tubers

The isolation of mitochondria was accomplished by using the method of Palmer (1967) with several modifications. Six hundred grams of peeled potato pieces were grated in 500 ml of a medium consisting of 0.5 M sucrose and 0.05 M Tris-HCl buffer (pH 7.8) with 10^{-5} M streptomycin. The homogenate was then stirred and filtered through four layers of cheesecloth and two layers of miracloth. The resulting solution was centrifuged at 3,000 g for 10 min to remove cellular debris and starch. The supernatant fluid was then centrifuged at 15,000 g for 25 min and the resulting pellet was suspended in 25 ml of 0.4 M sucrose and 0.05 M Tris-HCl buffer (pH 7.2) containing 10^{-5} M streptomycin. The mitochondria were then sedimented at 15,000 g for 25 min and finally suspended in 1.0 ml of 0.4 M sucrose and 0.05 M Tris-HCl buffer (pH 7.2) containing 10^{-5} M streptomycin and their respiratory activity was measured. All the solutions and apparatus used in the isolation procedures were maintained at 4°C. Respiration experiments were carried out in a Gilson Differential Respirometer. Each flask contained in 1.4 ml of active mitochondria (0.8 mg protein/reaction), 40 μM Pi, 2 μM ATP, 0.02 μM cytochrome C, and 20 μM of sodium succinate. For measuring the effect of the purified enzymes on respiratory activity of mitochondria, 0.4 ml of the purified enzyme preparation was added to the flask side arm and poured into the reaction medium after a given period of time. All of the experiments were carried out in a 25°C water bath.

Results

Enzyme activities—The activity of *endo*-polygalacturonate *trans*-eliminase

Table 1. *Ammonium sulfate fractionation of the crude culture filtrate of* ***Erwinia carotovora*** *(EC14)*[1]

% $(NH_4)_2SO_4$ Saturation[2]	Relative activity (pH 8.0)		
	Endo-PGTE	Phosphatidase C	Protease
0-40	22.0	22.4	49.0
40-60	22.8	100.0	61.0
60-80	23.6	19.4	100.0
80-90	100.0	0.0	0.0

(1) The culture was grown on autoclaved bean hypocotyls for 14 days at 30°C.
(2) Seven grams of crude lyophilized enzyme (9.6 mg protein/ml) were dissolved in 100 ml of distilled water and dialyzed at 4°C against distilled water for 24 hr. The dialyzed solution was stirred and powdered ammonium sulfate was added to 40, 60, 80, and 90% saturation at 23°C for 1 hr. The precipitate was collected by centrifugation at 20,000 g for 20 min at 4°C, The precipitate from each fraction was dissolved in 10 ml of cold distilled water, and the solution was immediately assayed for protein content and enzyme activities.

(*endo*-PGTE), phosphatidase C, and protease was in the alkaline range as was previously reported (Mount *et al.*, 1970; Tseng and Bateman, 1968). Endo-PGTE produced by *E. carotovora* exhibited a maximum enzyme activity on sodium polypectate at pH 8.0–8.5 and activity was stimulated by the addition of 10^{-4} M Ca^{2+}. The production and activity of protease was first estimated by utilizing various protein substrates—hemoglobin, casein hydrolysate, bovine serum albumin, and gelatin. The crude enzyme preparation contained a protease which hydrolyzed gelatin, casein hydrolysate, and bovine serum albumin but was not active on hemoglobin. Gelatin was the best substrates tested. Optimal enzyme activity was between pH 8.0 and 8.5. The enzyme was stimulated by Ca^{2+} (10^{-4} M) but was inhibited by Zn^{2+} (10^{-4} M) and Co^{2+} (10^{-4} M). The phosphatidase C produced by *E. carotovora* was active in the alkaline range (Mount and Bateman, 1969). The enzyme activity was stimulated by 10^{-4} M Mg^{2+} and 10^{-4} M Ca^{2+}.

Enzyme purification

Phosphatidase C—Phosphatidase C was mainly found in the precipitate obtained from the 40–60% ammonium sulfate $(NH_4)_2SO_4$ fraction (Table 1). This fraction was used for further purification of the enzyme on DEAE cellulose. As previously report (Tseng and mount, 1974), two peaks showing phosphatidase C activity were eluted on DEAE cellulose column. Since the first peak showing phosphatidase activity (phosphatidase I) had a higher specific activity (3.4) than the second (3.0), only phosphatidase I was used for further purification. This peak also contained protease and *endo*-PGTE.

Table 2. *Purification of phosphatidase C, protease, and endo-polygalacturonate trans-eliminase (endo-PGTE) from the culture filtrates of* ***Erwinia carotovora*** *(EC14)*

Enzyme	Fraction	Volume (ml)	Activity[1] (units/ml)	Specific activity (units/mg protein)	Purification (fold)
Phosphatidase C	1. Culture filtrate	100.0	26.0	2.7	—
	2. 40-60% $(NH_4)_2SO_4$	3.0	13.6	3.4	1.2
	3. DEAE cellulose chromatography	35.0	6.4	23.2	8.6
	4. Isoelectric focusing	15.0	7.6	63.2	23.4
Protease	1. Culture filtrate	100.0	18.5	2.8	—
	2. 60-80% $(NH_4)_2SO_4$	3.0	10.0	3.7	1.3
	3. DEAE cellulose chromatography	35.0	16.2	92.0	32.8
	4. Isoelectric focusing	15.0	24.0	160.0	57.1
Endo-PGTE	1. Culture filtrate	100.0	66.8	6.9	—
	2. 80-90% $(NH_4)_2SO_4$	3.0	56.0	50.0	7.2
	3. DEAE cellulose chromatography	25.0	63.8	240.0	34.7
	4. Isoelectric focusing	15.0	53.0	970.0	140.6

(1) A unit of *endo*-PGTE activity is defined as that amount of enzyme giving an increase in absorbance of 0.1 in 60 min at 548 nm using the thiobarbituric acid assay. Phosphatidase C activity is defined as the amount of enzyme that causes the release of 10 μg of acid-soluble phosphorus in 60 min at 30°C. Protease activity is defined as that amount of enzyme that causes an increase in absorbance of 0.01 at 280 nm per hr under the standard conditions as described in the test.

Phosphatidase C, protease, and *endo*-PGTE could not be separated by DEAE cellulose column chromatography. However, isoelectric focusing gave good separation of phosphatidase C, protease, and *endo*-PGTE with isoelectric points at pI 7.5, 8.3 and 9.4, respectively. A 23-fold increase in purity was obtained for phosphatidase C (Table 2).

Protease—When the crude culture filtrate was fractionated with ammonium sulfate, maximum protease activity was found in the precipitate obtained between 60-80% $(NH_4)_2SO_4$ saturation (Table 1). The precipitate was dissolved in 10 ml of distilled water, dialyzed and applied onto a DEAE cellulose column. Only one protease peak was eluted between 70-90 ml volume from the column (Fig. 1). Again, two phosphatidase C peaks were eluted with a elution profile

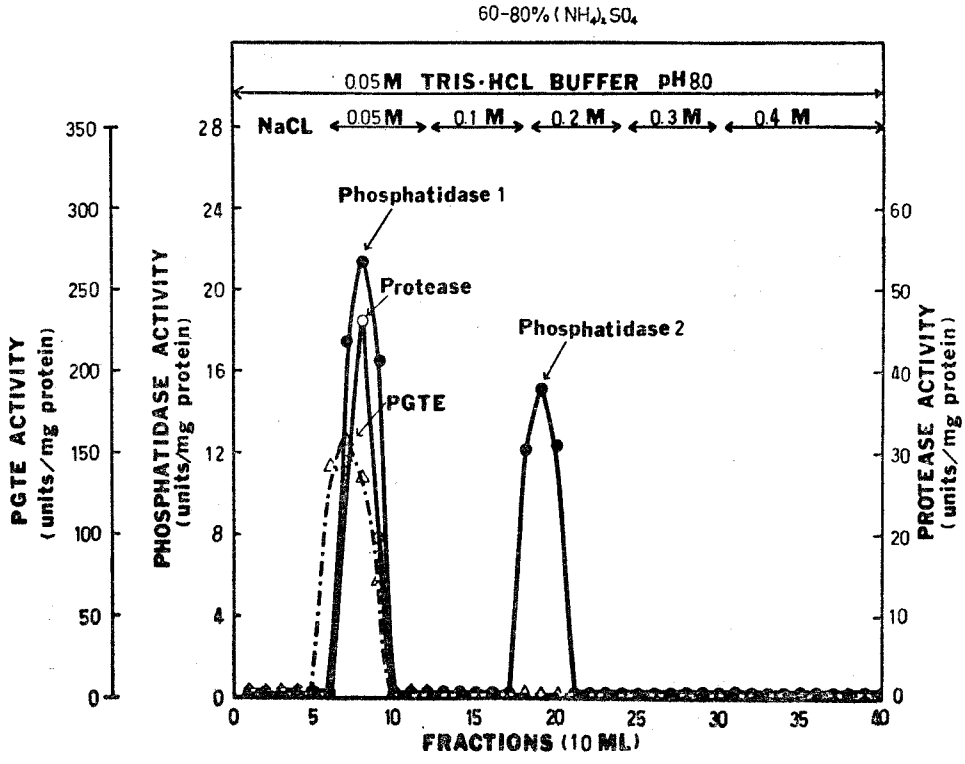

Fig. 1. DEAE cellulose column chromatography of dialyzed enzyme from the 60–80% ammonium sulfate saturation. The column was eluted with a NaCl stepwise gradient. Ten ml fractions were collected and activities of protease, phosphatidase C and *endo*-polygalacturonate *trans*-eliminase were immediately assayed.

similar to that obtained from the 40–60% $(NH_4)_2SO_4$ fraction. Isoelectric focusing of the 70–90 ml elute resulted in the complete separation of the three enzymes with isoelectric points similar to those from the 40–60% $(NH_4)_2SO_4$ fraction (Fig. 2). A 57-fold increase in purity was obtained for protease after isoelectric focusing (Table 2).

Endo-polygalacturonate *trans*-eliminase—The greatest activity of *endo*-PGTE was found in the 80–90% ammonium sulfate precipitate (Table 1). Only *endo*-PGTE was evident in this fraction. Further purification on DEAE cellulose (Fig. 3) and isoelectric focusing (Fig. 4) resulted in a 141-fold purification of *endo*-PGTE.

The purity of each enzyme from the electrofocusing column was carefully examined. The activity (units/mg protein) of each enzyme was adjusted in the following experiments in order that replication could be kept constant and interpretation of the results simplified.

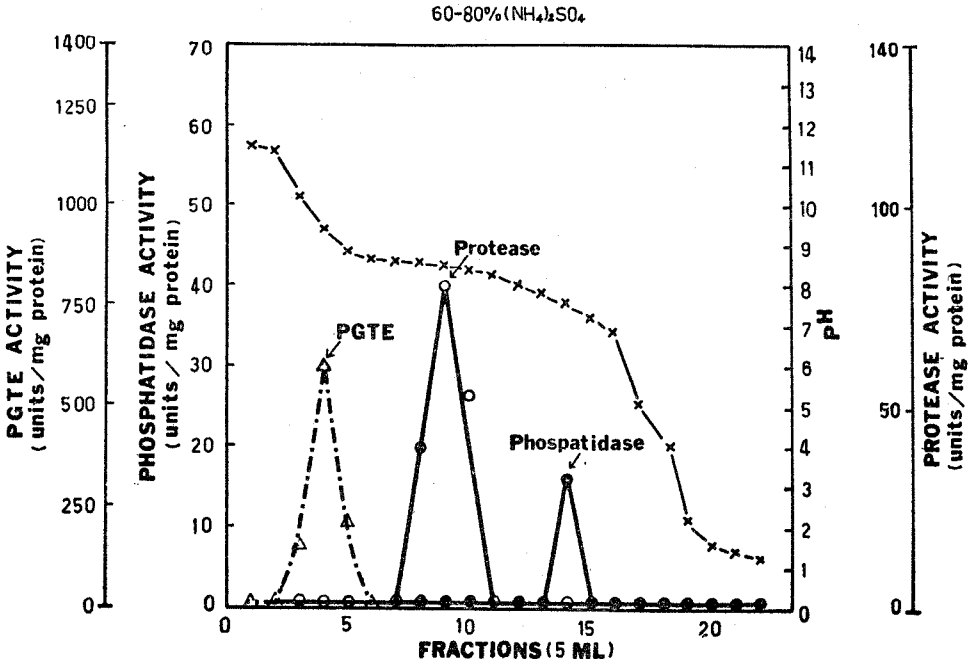

Fig. 2. Separation of protease, phosphatidase C and *endo*-polygalacturonate *trans*-eliminase by isoelectric focusing using ampholine carrier with a pH range of 7-10 at 5°C for 48 hr. The starting material was fractions 5-10 from the DEAE cellulose column described in Fig. 1.

Respiration of enzyme-treated potato discs and mitochondria

The isolated mitochondria were treated with the three purified enzymes and the effect of these enzymes on respiratory activity were measured (Fig. 5). The mitochondrial reaction mixture was allowed to equilibrate for 30 min in the respirometer flasks before the purified enzymes were tipped from the side arm of the flasks into reaction chamber. Only purified phosphatidase C and protease produced a decrease in oxygen uptake. The effect of phosphatidase on mitochondria was much greater than that of protease. No cumulative effect was observed when the two enzymes, phosphatidase C and protease, were combined. Purified *endo*-PGTE exhibited no appreciable effect on mitochondrial respirating activity.

Changes in the rate of oxygen uptake after potato discs were treated with the purified enzymes are illustrated in Fig. 6. In contrast to the results obtained from the enzyme-treated mitochondria, *endo*-PGTE caused a maximum decrease in respiration of potato discs in which oxygen uptake was reached after 5 hr incubation. The maceration of potato discs was periodically checked in duplicate flasks and maceration was complete by the fifth hour of incubation. Only slight decrease in oxygen uptake were observed when discs were treated

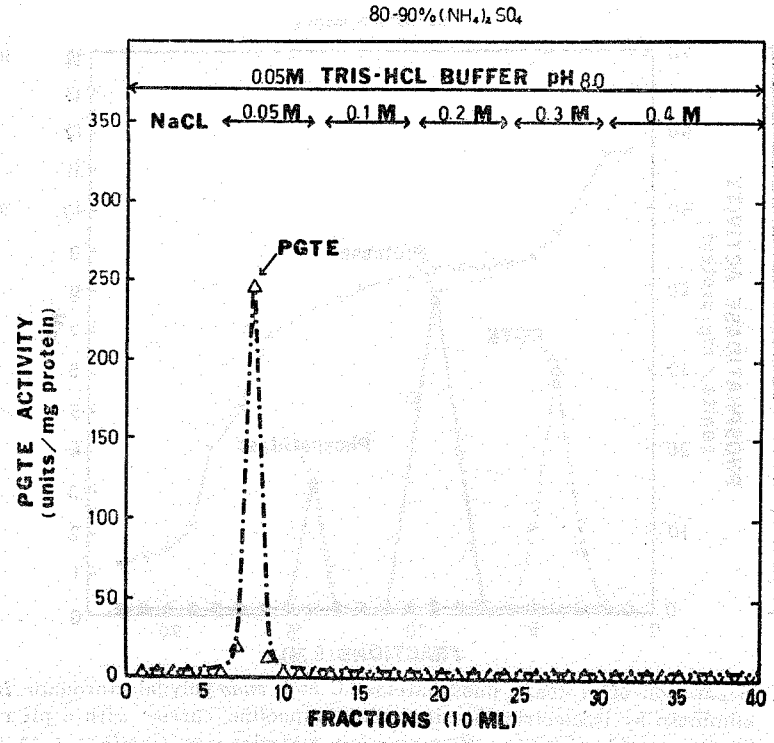

Fig. 3. Purification of *endo*-polygalacturonate *trans*-eliminase with DEAE cellulose column chromatography. Three ml of dialyzed enzyme from 80-90% ammonium sulfate saturation were applied to a DEAE cellulose column (2.5×23 cm) at 5°C. Ten ml fractions were collected and the enzyme activity was immediately assayed by the thiobarbituric acid method. No protease or phosphatidase C activity was detected at this level of $(NH_4)_2SO_4$ saturation.

with either phosphatidase C or protease.

Discussion

In order to understand the involvement of various enzymes produced by pathogens in disease development as well as their effects on host cellular organelles, highly purified enzyme systems must be used. In this study three enzymes, *endo*-PGTE, protease, and phosphatidase C, were selected for purification. Purification of these enzymes was accomplished by ammonium sulfate fractionation, DEAE cellulose column chromatography, and isoelectric focusing. Two phosphatidase fractions were eluted from DEAE cellulose column (Fig. 1). Both phosphatidases were found to be of the C type. The presence of two phosphatidase C fractions suggest that two isoenzymes were induced. Further studies using other methods such as polyacrylamide gel electrophoresis and/or sucrose density gradient ultracentrifugation are needed before confir-

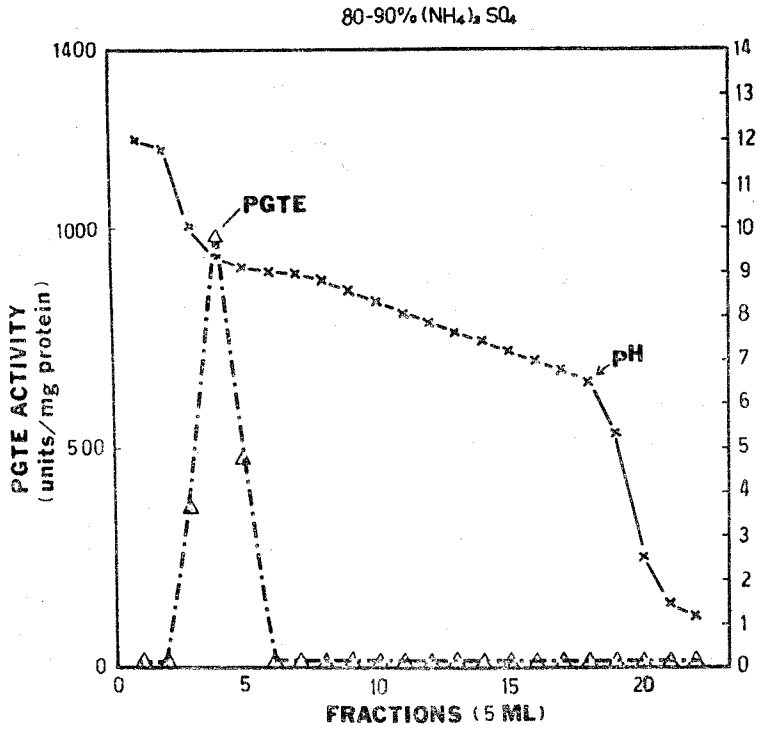

Fig. 4. Isoelectric focusing of *endo*-polygalacturonate *trans*-eliminase fractions from DEAE cellulose column chromatography (Fig. 3) using ampholine carrier with a pH range of 7–10 at 5°C for 48 hr. Five ml fractions were collected and immediately measured for pH values. After dialyzation at 4°C for 24 hr, each fraction was checked for enzyme activity with the thiobarbituric acid procedure.

mation of phosphatidase isoenzymes can be made. Several investigators have demonstrated the presence of multiple phosphatide-degrading enzymes of fungal and bacterial origin (Doery *et al.*, 1965; Hayaishi and Kornbery, 1954; Oi and Satomura, 1963). Recently, Salach *et al.* (1968) were able to isolate phospholipase A isoenzymes from Naja Naja venom.

Descriptions of alternations of respiration in plant tissues affected by extracellular enzymes of bacterial pathogens are meager. Research in this area has been primarily with the crown gall pathogen, *Agrobacterium tumefaciens* (Nagy, 1938), and with *Pseudomonas solanacerum* (Maine, 1960). When partially purified hydrolytic enzymes (polygalacturonase and cellulase) from culture filtrates of *P. solanacerum* were incubated with tobacco stem slices, O_2 uptake by susceptible stem slices was increased 58 percent. No increase in O_2 uptake was observed when resistant stem slices were tested. In the present study, highly purified *endo*-PGTE from culture filtrates of *E. carotovora*, when incubated with potato discs, resulted in a significant decrease in O_2 uptake (Fig. 6).

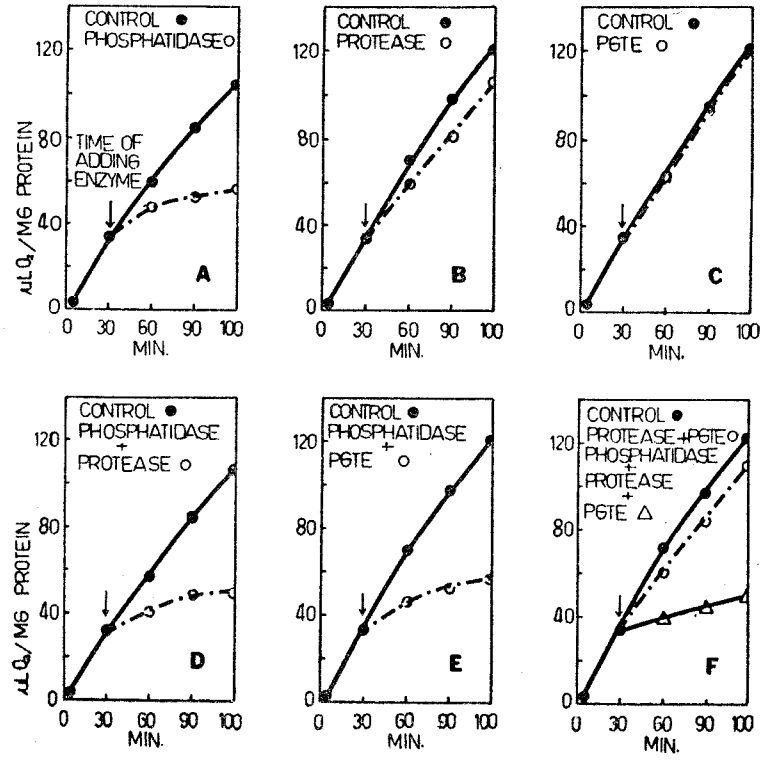

Fig. 5. Oxygen uptake by potato mitochondria treated with purified phosphatidase C, protease, and *endo*-polygalacturonate *trans*-eliminase produced by ***Erwinia carotovora*** (EC14). Each flask contained in 1.4 ml of active mitochondria (0.8 mg protein/reaction), 40 μM Pi, 2 μM ATP, 0.02 μM cytochrome C, 20 μM of sodium succinate and 10^{-5} M of streptomycin. Four tenths ml of the purified enzyme preparation was added to the side arm and poured into the reaction medium after 1 hr incubation. All of the experiments were carried out at 25°C with 30 min equilibration before initial readings were taken. Mitochondria treates with phosphatidase C (3.3 units) with 3×10^{-4} M Mg^{2+} (A); Mitochondria treated with protease (6.6 units) with 3×10^{-4} M Ca^{2+} (B); *Endo*-polygalacturonate *trans*-eliminase (13.3 units) with 3×10^{-4} M Ca^{2+} (C); Protease (6.6 units) and phosphatidase C (3.3 units) with each 3×10^{-4} M Mg^{2+} and Ca^{2+} (D); Phosphatidase C (3.3 units) and *endo*-polygalacturonate *trans*-eliminase (13.3 units) with each 3×10^{-4} M Mg^{2+} and Ca^{2+} (E); Protease (6.6 units), phosphatidase C (3.3 units) and *endo*-polygalacturonate *trans*-eliminase (13.3 units) with each 3×10^{-4} M Ca^{2+}, Mg^{2+} and Ca^{2+}, respectively (F). Autoclaved enzymes were served as the controls.

No appreciable differences in O_2 uptake were found when purified phosphatidase C and protease were tested. Since *endo*-PGTE did not burst protoplasts and had no effect on mitochondrial respiration, one could speculate that the decrease in O_2 uptake of treated potato discs is due to an indirect effect. The decrease in O_2 uptake by mitochondria treated with phosphatidase C or protease may

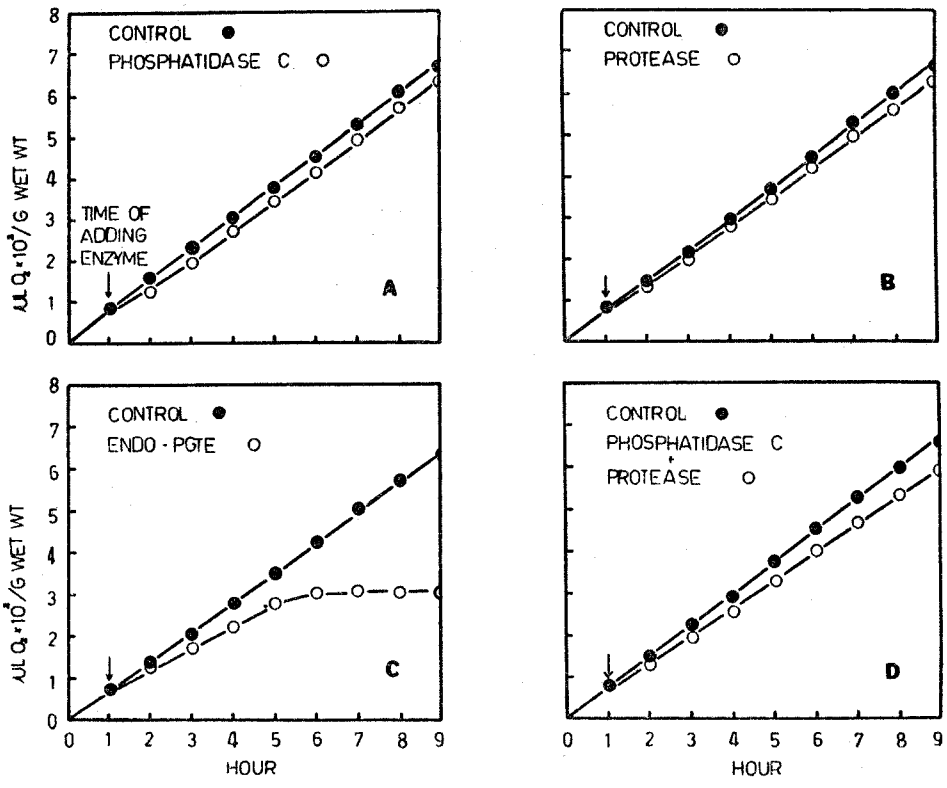

Fig. 6. Oxygen uptake by potato discs treated with purified phosphatidase C, protease and *endo*-polygalacturonate *trans*-eliminase produced by *Erwinia carotovora* (EC14). Each flask contained 40 μM Pi, 2 μM ATP, 0.02 μM sodium succinate and 0.2 g of small potato discs with 10^{-6} M streptomycin. All of the experimental conditions were the same as those described in Fig. 5. Potato discs treated with phosphatidase C (6.6 units) (A); Potato discs treated with protease (13.2 units) (B); Potato discs treated with *endo*-polygalacturonate *trans*-eliminase (26.6 units) (C); Potato discs treated with phosphatidase C (6.6 units) and protease (13.2 units) (D).

be explained several ways. These enzymes may act directly on the membrane to modify mitochondrial stability or indirectly by releasing membrane-bound enzymes which in turn directly interfere with the respiratory process. Phosphatidase C may split phosphatide molecules which are associated with certain respiratory enzymes. The alpha toxin of *Clostridium welchii* inhitits succinate oxidase (McFarlane, 1950; Wooldridge and Higginbattom, 1938) and cytochrome oxidase activities of mitochondrial preparations (McFarlane, 1950). In addition, lecithin is also important in maintaining the activity of an ATPase (Kielley and Meyerhof, 1950) in which a linear correlation between the amount of acid-soluble phosphorus released by toxin action and the inhibition of ATPase has been established. The release of acid-soluble phosphorus parallels a decrease

in succinate activity (Edwards and Ball, 1954). Augustyn *et al.* (1970) reported that phospholipase A from *Bgkistrodon piscivorus* alters mitochondrial respiration and phosphorylation in a manner identical to whole venom: at low concentration it increases mitochondrial respiration in the absence of a phosphate acceptor (ATP); at high concentration it causes inhibition of the electron transport system; and at intermediate concentrations it produces a respiratory rate decline in which ADP acts in a direct or indirect manner in causing a decrease in respiratory activity remains to be investigated.

The fact that both purified phosphatidase C and protease caused bursting of cucumber protoplasts (Tseng and Mount, 1974) and altered mitochondrial respiration, indicated a modification of cellular membranes of host.

Acknowledgment

The author is greatly indebted to Dr. M. S. Mount, Professor of Plant Pathology, University of Massachusettes for his criticism during the preparation of the manuscript.

Literature cited

AUGUSTYN, J. M., B. PARSA and W. B. ELLIOTT. 1970. Structural and respiratory effects of *Agkistrodon piscivorus* phospholipase A on rat liver mitochondria. Biochem. Biophys. Acta **197**: 185-196.

DOERY, H. M., B. J. MAGNUSSON, J. GULASEKHARAM and J. E. PEARSON. 1965. The properties of phospholipase enzymes in *Staphylococal* toxins. J. Gen. Microbiol. **40**: 238-296.

EDWARDS, S. W. and E. G. BALL. 1954. The action of phospholipases on succinate oxidase and cytochrome oxidase. J. Biol. Chem. **209**: 619-633.

GARIBALDI, A. and D. F. BATFMAN. 1971. Pectic enzymes produced by *Erwinia chrysanthemi* and their effects on plant tissue. Physiol. Plant Path. **1**: 25-40.

HAYAISHI, O. and A. KORNBERG. 1954. Metabolism of phospholipids by bacterial enzymes. J. Biol. Chem. **296**: 647-663.

KIELLEY, W. W. and O. MEYERHOF. 1950. Studies on adenosine-triphosphatase of muscle. III. The lipoprotein nature of the magnesium-activated adenosine-triphosphatase. J. Biol. Chem. **183**: 391-401.

KUNITZ, M. 1974. Crystalline soybean inhibitor. II. General properties. J. Gen. Physiol. **30**: 291-310.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**: 265-275.

LUMSDEN, R. D. and D. F. BATEMAN. 1968. Phosphatide-degrading enzymes associated with pathogenesis in *Phaseclus vulgaris* infected with *Thielaviopsisid basicola*. Phytopathology **58**: 219-237.

MAINE, E. C. 1960. Physiological responses in the tobacco plant to pathogensis by *Pseudomonase solanacerum*, causal agant of bacterial wilt. Doctoral Thesis, North Carolina State College, Durham, N. C. 98 p.

MCFARLANE, M. 1950. Inhibition of succinoxidase activity of mitochondria by *Clostrium welchii* toxin. Biochem. J. **47**: xxix-xxx.

MOUNT, M. S. and D. F. BATEMAN. 1969. Production of phosphatidase C by *Erwinia carotovora* Phytopathology **59**: 401 (Abstr.).

MOUNT, M.S. and D.F. BATEMAN and H.G. BASHAM. 1970. Induction of electrolyte less, tissue maceration, and cellular death of potato tissue by an *endo*-polygalacturonate *trans*-eliminase. Phytopathology **60**: 924-931.

NAGY, R., A.J. RIKER and W.H. PETERSON. 1938. Some physiological studies on crown gall and contiguous tissue. J. Agr. Res. **57**: 465-472.

OJ, SUSUMU and Y. SATOMURA. 1963. Purification and substrate specificates of phospholipase A and B of *Sclerotinia* fungus. Agr. Biol. Chem. **27**: 397-404.

PALMER, J.M. 1967. Rapid isolation of active mitochondria from plant tissue. Nature **216**: 1208.

PREISS, J. and G. ASHWELL. 1963. Polygalacturonic acid metabolism in bacteria. I. Enzymatic formation of 4-deoxy-L-threo-5-hexoseulose uronic acid. J. Biol. Chem. **238**: 1571-1576.

SALACH, J.I., P. TURINI, J. HAUBER, R. SENG, H. TISDALE and T.P. SINGER. 1968. Isolation of phospholipase A isoenzymes from *Naja Naja* venom and their action on membrane-bound enzymes. Biochem. Biophys. Res. Commun. **33**: 936-941.

TSENG, T.C. and L.H. CHANG. 1974. *Sclerotium rolfsii* phosphatidase B. I. Inhibition of mung bean mitochondrial oxygen uptake by the phosphatidase. Bot. Bull. Academia. Sinica **15**: 8-13.

TSENG, T.C. and M.S. MOUNT. 1974. Toxicity of *Endo*-polygalacturonat *trans*-eliminase, phosphatidase and protease to potato and cucumber tissue. Phytopathology **64**: 229-236.

TURNER, M.T. and D.F. BATEMAN. 1968. Maceration of plant tissues susceptible and resistant to soft rot pathogens by enzymes from compatible host-pathogen combinations. Phytopathology **58**: 1509-1515.

WOOLDRIDGE, W.G. and C. HIGGINBOTTOM. 1938. The effect of certain bacterial toxins upon some respiratory mechanisms of animal tissues. Biochem. J. **32**: 1718-1728.

軟腐細菌所分泌之果膠質分解酵素，磷脂分解酵素和蛋白分解酵素之純化以及對馬鈴薯粒腺體呼吸作用之研究

曾 聰 徹

中央研究院植物研究所

軟腐細菌所分泌之三種酵素，果膠質分解酵素，C型磷脂分解酵素和蛋白分解酵素經硫酸氨分劃法，DEAE 纖維色層分析法和等電點分劃法，將該酵素分別純化爲23倍，57倍和141倍，並求得各酵素之等電點分別爲 7.5，8.3和 9.5。

純化之蛋白質分解酵素和C型磷脂分解酵素都具有抑制馬鈴薯粒腺體呼吸作用之功能，其中以磷脂分解酵素之抑制率較高，而果膠質分解酵素並不抑制對粒腺體之呼吸作用。相反地，當使用馬鈴薯組織切片爲研究材料去做相同試驗時，發現果膠質分解酵素對抑制呼吸作用率却高於其他兩種純化酵素。