Toxicity of Endopolygalacturonate trans-eliminase, Phosphatidase and Protease to Potato and Cucumber Tissue

T. C. Tseng and M. S. Mount

Research Assistant and Assistant Professor, respectively, Department of Plant Pathology, University of Massachusetts, Amherst 01002. Present address of senior author: Institute of Botany, Academia Sinica, Nankang, Taipei, Taiwan.

A portion of this work was submitted by the senior author in partial fulfillment of the requirements for the Ph.D. degree from the University of Massachusetts, Amherst.

Supported in part by NSF Grant No. GB-22626.

Accepted for publication 19 September 1973.

ABSTRACT

Three extracellular enzymes [endopolygalacturonate *trans*-eliminase (endo-PGTE), phosphatidase C, and protease] produced by *Erwinia carotovora* were purified by ammonium sulfate fractionation, diethylaminoethyl cellulose column chromatography, and isoelectric focusing. Maceration of potato and cucumber disks occurred only when purified endo-PGTE was present in the reaction mixture. No cellular death was evident when potato tissue was treated with phosphatidase and protease, however, some cellular injury was evident when cucumber was used as the substrate. Only endo-PGTE induced leakage of ^{86}Rb from potato disks. No leakage of ^{86}Rb occurred from cucumber tissue when treated with any of the purified enzymes. Both phosphatidase and protease caused isolated cucumber protoplasts to burst, whereas endo-PGTE did not. Leakage of neutral red from the protoplasts was observed before, and at the time of bursting of, phosphatidase- and protease-treated protoplasts. These results suggest that endo-PGTE has no apparent effect on the membranes of cucumber protoplasts, and that phosphatidase and protease of *E. carotovora* may play a role in disease development.

Phytopathology 64:229-236

Additional key words: permeability change, Ampholine electrofocusing.

Purified endo-polygalacturonate *trans*-eliminases from *Erwinia carotovora* and *E. chrysanthemi* are capable of causing electrolyte loss, maceration, and cellular death of potato tissue (11, 16). The rate of host cell death parallels the rate of maceration (5, 10, 16, 22, 23). However, the mechanism of the killing action by pectic enzymes is unknown. Since cellular death of plasmolyzed tissue, which has been reacted with pectic enzymes, can be delayed in relation to the rate of maceration (23), the possibility exists that an appropriate substrate for the action of these enzymes may be present within the plant cell membrane or protoplasm (16). Observations of alkaline hydroxylamine and ferric chloride-stained onion cells suggests that pectic materials may be present in the cytoplasm (1). It has also been suggested that certain basic proteins (enzymes) may be toxic to plant cells by causing an imbalance of ionic sites on the cell membrane (19, 20). Evidence presented by Garibaldi and Bateman (11) showed that several pectic isoenzymes, purified from *E. chrysanthemi,* exhibited isoelectric points at and above pH 7.9 and caused cellular death. One of their pectic enzymes, which did not readily cause cellular injury, had an isoelectric point of pH 4.5. Bateman (4), however, has purified an endo-polygalacturonase (pI = 5.2) from *Sclerotium rolfsii* which macerates and kills host tissue.

In addition to endo-polygalacturonate *trans*-eliminase, *E. carotovora* also produces large amounts of protease (9) and phosphatidase C (15, 16). Many phytopathogens produce phosphatidases (24) and it has been suggested that these enzymes may act as toxic agents (23) and may be responsible for killing of plant tissues. Lumsden and Bateman (14) observed an association of phosphatidase(s) in bean tissues infected with *Thielaviopsis basicola*. Friedman (9) suggested that increased pathogenicity of *E. carotovora* may be due to greater protease activity which he found associated with a virulent strain of this bacterium. Since proteins and phospholipids are major components of cell membranes (6), the involvement of proteases and phosphatidases in pathogenesis cannot be overlooked.

An essential prerequisite for understanding the true enzymatic nature of host injury by pathogens is the use of highly purified enzyme systems. Although pectic enzymes have been highly purified and tested against various intact plant tissues (4, 11, 16), there is very little information on the effects of purified phosphatidases or proteases, and on the role these enzymes play in pathogenesis.

The question of interest in the phenomenon of cellular death induced by pectic enzymes is whether purified endo-polygalacturonate *trans*-eliminase will cause ion leakage or cellular death in the absence of the cell wall. In other words, would the cell membrane or protoplasm be sufficient substrates for the toxic action of this enzyme? Also, in the same context, would purified proteases and/or phosphatidases cause chemical disruption of plant protoplasts, and does the cell wall act as a barrier against the action of these enzymes?

The objectives of this investigation were: (i) to isolate and purify enzymes (phosphatidases, protease, and endo-polygalacturonate *trans*-eliminase) produced by *E. carotovora;* and (ii) to ascertain the effects of these enzymes upon cucumber and potato tissues in relation to the phenomena of maceration, cellular death (intact plant tissue versus isolated protoplasts), and changes in cell membrane permeability.

MATERIALS AND METHODS.—Isolate EC14 of *Erwinia carotovora* (Jones) Holland, used in a previous study (16), was maintained on nutrient agar slants at 25 C.

Preliminary studies were performed to select a suitable medium for the production of phosphatidase, protease, and endo-polygalacturonate *trans*-eliminase (endo-

PGTE). The media tested were: (i) Nutrient broth (Difco) supplemented with 0.5% sodium polypectate (Sunkist Growers, Ontario, Calif.); (ii) Nutrient broth supplemented with 0.5% sodium polypectate plus potato tuber extracts (100 g/liter); (iii) Nutrient broth supplemented with 0.5% sodium polypectate plus 0.1% lecithin (Sigma); (iv) Sliced cucumber fruits (300 g/liter); and (v) 7-day-old bean hypocotyls (*Phaseolus vulgaris* L. 'Red Kidney') as used in a previous study (24). The media were autoclaved and incubated with *E. carotovora* for 14 days at 30 C. Culture filtrates from the liquid cultures were harvested by centrifugation at 20,000 *g* for 20 min at 4 C, and the supernatants lyophilized and assayed for enzyme activities. Bacterial cultures which were grown on bean hypocotyls and sliced cucumber media were harvested by blending the flask contents with 50 ml of distilled water in a Virtis '45' homogenizer for 1 min at high speed. The extracts were filtered through four layers of cheesecloth to remove debris and then centrifuged at 20,000 *g* for 20 min at 4 C. Again, the supernatant was lyophilized and assayed for enzyme activities. All media were good substrates for enzyme production by *E. carotovora;* however, the phosphatidase which was extracted from media (i) through (iv) was somewhat unstable and rapidly lost its activity after purification. The enzymes from culture filtrates of *E. carotovora* grown on the bean hypocotyl medium were highly stable after purification (active after 1 yr) and served as the main source of enzymes for this study.

Enzyme assays.—Since the phosphatidase produced by *E. carotovora* has been identified as a phosphatidase C (15), the enzyme activity was quantitatively assayed by measuring the release of acid-soluble phosphorus (phosphorylcholine) from soybean lecithin, as described by Lumsden and Bateman (14). 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 enzymes, 0.5 ml of 0.05 M Tris-HCl buffer (pH 8.0), and 0.25 ml of 0.025 M $CaCl_2$. Reaction mixtures were incubated for 1 h at 30 C after which time 0.1 ml of 5% bovine serum albumin and 0.9 ml of 20% trichloroacetic acid (TCA) were added to stop enzyme activity. One unit of enzyme activity was defined as the amount of enzyme required to release 10 μg of acid-soluble phosphorus in 1 h at 30 C. The "cup plate" assay used as previously described (8) was employed for qualitative detection of phosphatidase activity.

The quantitative assay for protease activity was performed spectrophotometrically. Preliminary investigations indicated that the enzyme preparation contained a protease which hydrolyzed gelatin, casein hydrolysate, and bovine serum albumin, but did not hydrolyze hemoglobin. Among those substrates, gelatin was considered to be the best substrate for determining the protease activity. Reaction mixtures consisted of 1.0 ml of 1.0% (w/v) gelatin (Nutritional Biochemical Corp.) in 0.05 M 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 h. At the end of the incubation period 3.0 ml of 20% TCA was added and the contents thoroughly mixed. This solution was then centrifuged at 5,000 *g* for 20 min at room temperature. The absorbance of the clear supernatant, containing TCA - soluble hydrolysis products, was determined at 280 nm in a Beckman DB-G Spectrophotometer against a reaction blank. The reaction blank was identical to the reaction mixtures, except that the 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 of the TCA-soluble reaction products.

Endo-polygalacturonate *trans*-eliminase was measured by the periodate-thiobarbituric acid (TBA) method (18) in which the activity of the enzyme is indicated by the formation of a red chromagen which shows a maximum absorption at 548 nm. The reaction mixture contained 0.1 ml of 0.6% (w/v) sodium polypectate in 0.05 M Tris-HCl buffer (pH 8.0) plus 10^{-4} M $CaCl_2$ and 0.1 ml enzyme. The reaction mixture was incubated for 1 h at 30 C. One unit of endo-PGTE activity was defined as that amount of enzyme giving an increase in absorbance of 0.1 in 1 h at 548 nm.

All of the controls contained autoclaved enzymes instead of active enzymes except the control for the protease assay using the spectrophotometric method. Protein concentration was determined by the method of Lowry et al. (13). Crystalline bovine albumin was used as the reference protein.

Enzyme purification.—Seven grams of lyophilized culture extracts of *E. carotovora* (grown on bean hypocotyls) were dissolved in 100 ml of distilled water and dialyzed against several liters of cold distilled water for 24 h at 4 C. The dialyzed solution was brought to 40% saturation with powdered ammonium sulfate, allowed to stand for 1 h at 4 C, and centrifuged for 20 min at 20,000 *g*. The precipitate was collected and 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 phosphatidase C, protease, and endo-PGTE. Each fraction was dialyzed and separately subjected to diethylaminoethyl cellulose (DEAE cellulose, Cl^- form) column chromatography. The column size was 2.5 × 23 cm and the DEAE cellulose was equilibrated with 0.05 M Tris-HCl buffer, pH 8.0. The column was eluted with 60 ml of 0.05 M Tris-HCl at pH 8.0, followed by a step-wise NaCl gradient in buffer until 0.4 M NaCl was reached. Ten ml fractions were collected and assayed for enzyme activities.

The fractions from the DEAE cellulose elution, containing the three different enzymes, were combined and dialyzed for 24 h 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 a pH 7-10 Ampholine carrier (11, 16, 27). After electro-focusing, 5-ml fractions were collected and the pH of each fraction was immediately measured.

Tissue maceration and cellular death measurements.—Tissue maceration was evaluated and rated as described by Bateman (3) and Mount et al. (16). Disks (8.0 mm × 0.2 mm) of potato and cucumber fruit tissues served as the substrate in all of these studies. Disks were placed in enzyme preparations buffered at pH 8.0 with 0.05 M Tris-HCl buffer. Measurements of tissue maceration were made over a 4-h incubation period with

the various enzymes. Disks which did not macerate received a rating of 0, while those that were completely macerated received a score of 5. Cellular death was determined using the method of Tribe (23) in which the degree of retention of neutral red within the disks (protoplasts) was a measurement of cellular death. Reaction mixtures were the same as those described for tissue maceration. Cells (protoplasts) which retained the dye were considered alive and were scored 0. Cellular death was indicated by the absence of stained protoplasts and these disks received a rating of 5. Intermediate values were assigned to disks which exhibited various degrees of maceration and neutral red retention.

Measurement of permeability change.—The permeability change of potato and cucumber tissues treated with enzymes was measured by determining the loss of ^{86}Rb from tissues which had accumulated ^{86}RbCl (16). Potato and cucumber disks were allowed to take up ^{86}Rb in a buffered solution (0.05 M Tris-HCl, pH 8.0) containing 20 μc ^{86}Rb (1.7 μc ^{86}Rb/mg RbCl). Eight disks with dimensions of 8 mm × 0.4 mm were put into test tubes containing 2.0 ml of enzyme and 2.0 ml of 0.05 M Tris-HCl buffer (pH 8.0) and incubated at 30 C for various periods of time. Autoclaved enzymes or Tris-HCl buffer substituted for active enzymes were used for the controls. At time zero, and various intervals thereafter, 5 μl of bathing solution was removed and put into a scintillation counting vial. Fifteen ml of scintillation fluid (Liquifluor, New England Nuclear, in toluene) were added to each vial, and the radioactivity was measured with a Nuclear Chicago Liquid Scintillation Counter (Model 6850).

Isolation of cucumber protoplasts.—Protoplasts were only prepared from cucumber fruit locular tissues. Potato protoplasts were not studied in this work due to interference of starch granules and the low numbers of protoplasts obtained. Several pieces of the cucumber locular tissue were treated with 10% (w/v) pectinase (Nutritional Biochemical Corp.) and Celluase '36' (Rohm and Haas Co.) in 20% (w/v) sucrose and 0.025 M phosphate buffer, pH 7.3 for 30 min at 30 C. The mixture was strained through six layers of cheesecloth to remove

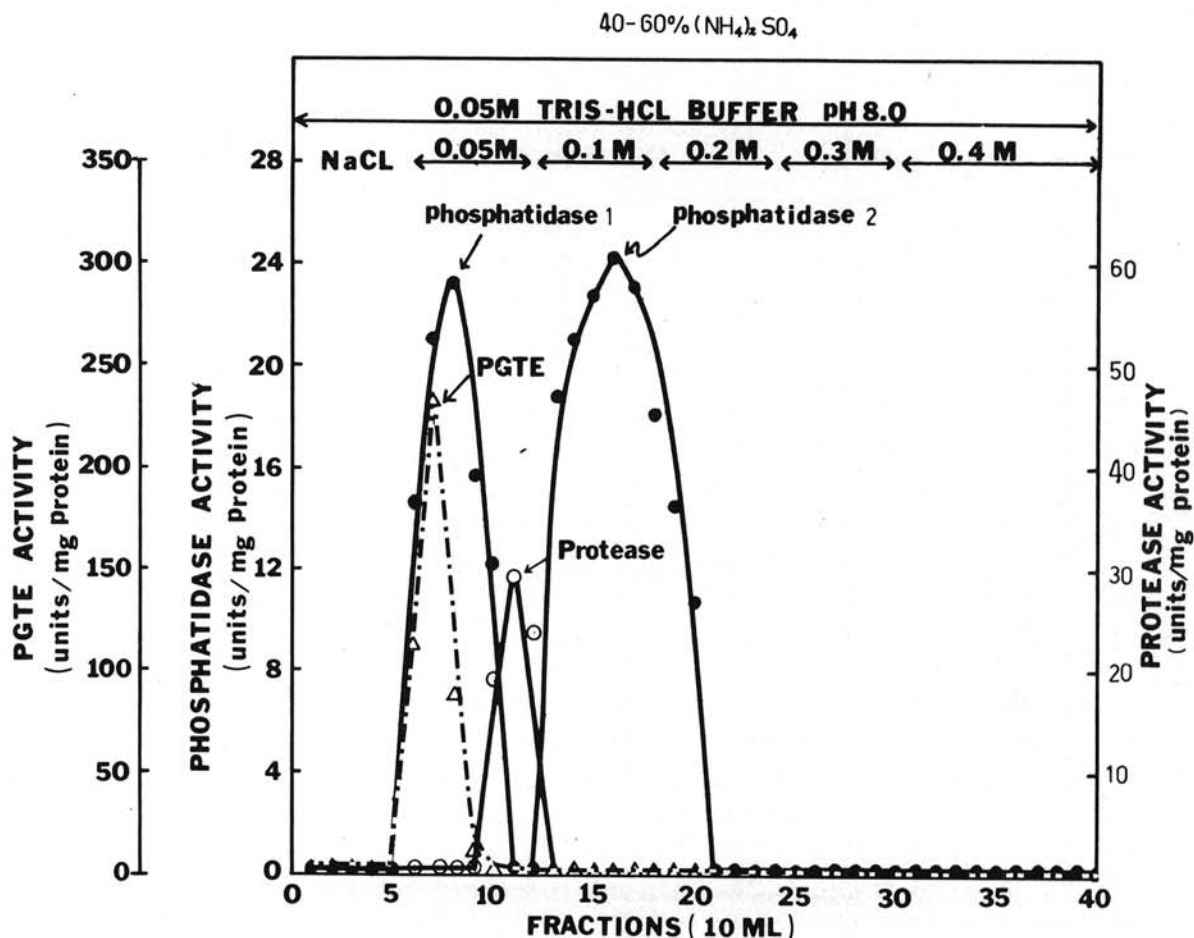

Fig. 1. Elution profile of protease, phosphatidase C, and endopolygalacturonate *trans*-eliminase from *Erwinia carotovora* by diethylaminoethyl (DEAE) cellulose column chromatography. Three ml of dialyzed enzyme from the 40-60% ammonium sulfate saturation were applied to the DEAE cellulose column (2.5 × 23 cm) at 4 C. Fractions (10.0 ml) were collected and enzyme activities were immediately assayed.

cellular debris. The filtrate, which contained protoplasts in suspension, was left for 5 min to allow any small debris to settle. The protoplasts, which floated upward and concentrated at the surface, were transferred to test tubes. Approximately 3.0 ml of the concentrated suspension of protoplasts were transferred to test tubes containing 0.6 M sucrose with 0.01% neutral red. After 40 min, the protoplasts which had floated to the surface were transferred as a concentrated suspension (approximately 3.0 ml) to another comparable test tube and the procedure was repeated. The repeated washings diluted the pectinase and cellulase 100-fold or more and served to dilute any contaminating enzymes from the locular juice. Fifty μl of the protoplast solution (containing ca. 250-500 protoplasts) were transferred to flat-bottom depression slides (20). The isolated protoplasts were then reacted immediately with an equal volume of the purified enzymes in 0.05 M Tris-HCl buffer (pH 8.0) with 0.6 M sucrose and the effects of these enzymes on the protoplasts were observed under the phase microscope.

RESULTS.—*Purification.*—Phosphatidase C, protease, and endo-PGTE were found in various concentrations (activity) in the 40-60 and 60-80% $(NH_4)_2SO_4$ fractions. Only PGTE activity was detected in the 80-90% fraction. All $(NH_4)_2SO_4$ fractions were saved and used for further purification on DEAE cellulose columns and by isoelectric focusing. Figure 1 represents a typical elution profile of the three enzymes from the 40-60% $(NH_4)_2SO_4$ fraction, on DEAE cellulose. The same elution profile was obtained by chromatography of the 60-80% fraction. Two phosphatidase peaks were always obtained. Only phosphatidase "peak-1" was used for further purification. This peak also contained protease and endo-PGTE. The enzymes within the 60-110 ml eluate (fractions 6 to 11) from the DEAE cellulose column were pooled, dialyzed, and subjected to isoelectric focusing using an Ampholine carrier which had a pH range of 7-10 (Fig. 2). Isoelectric focusing resulted in the separation of phosphatidase C, protease, and endo-PGTE with isoelectric points at 7.5, 8.3, and 9.4, respectively. The same isoelectric points of these enzymes were obtained when purification was carried out using the 60-80% $(NH_4)_2SO_4$ fraction. Final purification of these enzymes using the 40-60% $(NH_4)_2SO_4$ fraction is illustrated in Table 1. Purified enzymes used in the following experiments on maceration, cellular death, leakage of ^{86}Rb, and the bursting of cucumber protoplasts were the enzymes from the isoelectric focusing column (phosphatidase C, fraction 12; protease, fractions 7-10; and endo-PGTE, fractions 2-5) (Fig. 2).

Tissue maceration and cellular death.—The association of purified enzymes with tissue maceration

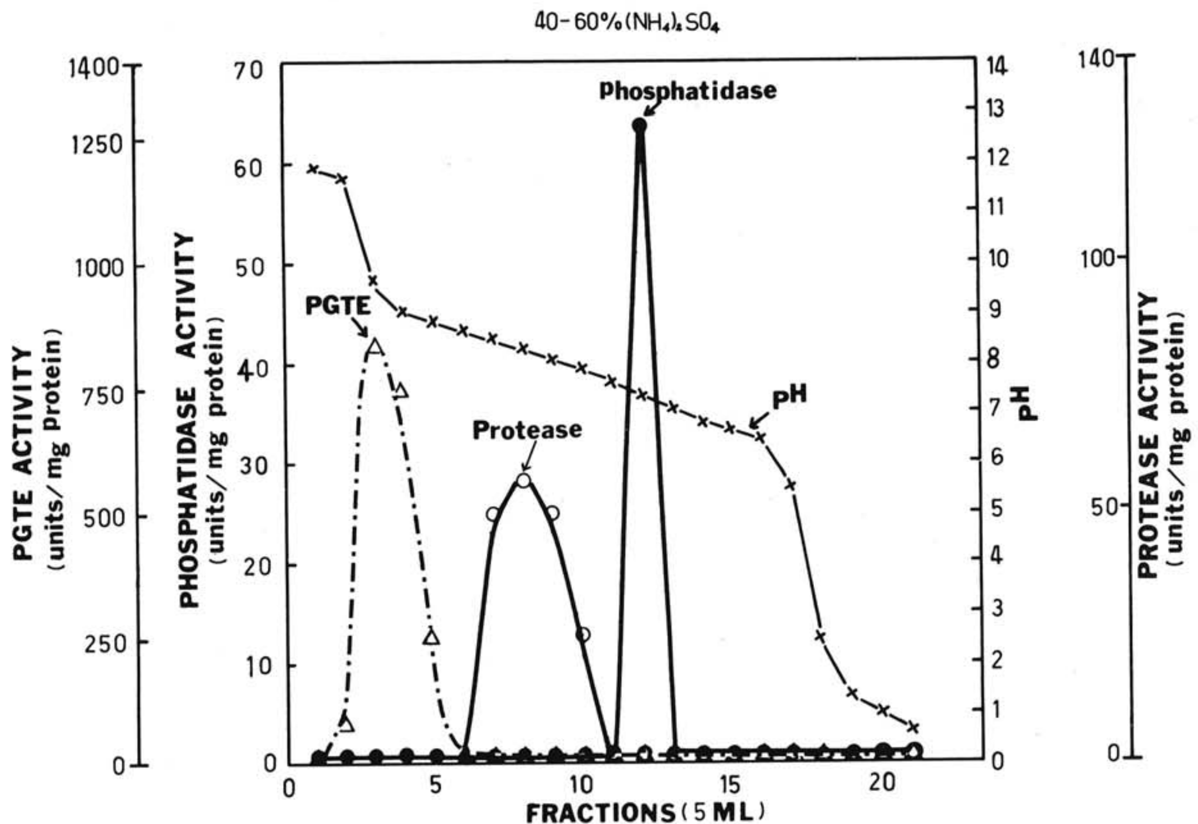

Fig. 2. Resolution of protease, phosphatidase C, and endopolygalacturonate *trans*-eliminase from fractions 6-11 eluted from the diethylaminoethyl (DEAE) cellulose column described in Fig. 1. Separation was accomplished by isoelectric focusing using Ampholine carriers with a pH range of 7-10 at 4 C for 48 h. Fractions (5.0 ml) were collected and immediately measured for pH values. After dialysis at 4 C for 24 h, each fraction was checked for enzyme activities.

TABLE 1. Final purification of phosphatidase C, protease, and endopolygalacturonate *trans*-eliminase (endo-PGTE) from culture filtrates of *Erwinia carotovora* through isoelectric focusing[a]

Enzyme	Units/mg protein[b]	Purification
Phosphatidase C		
1. Culture Filtrate	2.7	---
2. Isoelectric Focusing (fraction 12)	63.0	23.3
Protease		
1. Culture Filtrate	3.0	---
2. Isoelectric Focusing (fractions 7-10)	172.0	57.3
Endo-PGTE		
1. Culture Filtrate	5.8	---
2. Isoelectric Focusing (fractions 2-5)	1930.0	333.0

[a]This table represents the final purification of the three enzymes using the 40-60% $(NH_4)_2SO_4$ fraction as illustrated in Fig. 1 and 2.

[b]Definitions of units for each enzyme are described in Materials and Methods.

and cellular death is illustrated in Table 2. Maceration of both potato and cucumber disks occurred only when endo-PGTE was present in the reaction mixture. The rate of cellular death of potato disks paralleled the rate of maceration. No cellular death was evident when phosphatidase C or protease, alone or together, were incubated for 4 h with potato tissue. A cellular death index of 2 and 1 for phosphatidase C and protease, respectively, was obtained, however, for cucumber tissue after a 4-h incubation period. The effect of combining the enzymes after 3 h of incubation resulted in only a slight increase in cellular death (index of 2 as compared to an index of 1 for both phosphatidase C- and protease-treated cucumber disks).

Leakage of ^{86}*Rb*.—Potato and cucumber disks, which were allowed to accumulate ^{86}RbCl, were treated with the various purified enzymes. Only purified endo-PGTE readily induced leakage of ^{86}Rb from potato disks (Fig. 3-A). The loss of isotope from tissue treated with phosphatidase, protease, or a combination of the two enzymes was essentially the same as that obtained with autoclaved enzymes or buffer alone. When labelled cucumber tissues were treated with the purified enzymes, no significant differences were observed between the active enzymes and the control treatments (Fig. 3-B), although endo-PGTE has the ability to completely macerate and kill cucumber tissue within 1 h (Table 2). The radioactivity of a single cucumber disk was around 25×10^3 cpm indicating that the ^{86}Rb was accumulated within the tissue. The reasons for ^{86}Rb being released from endo-PGTE-treated potato tissue, but not from similarly treated cucumber tissue are not understood, but this may be an indication of the isotope being irreversibly bound to some component of the cucumber tissue. These results indicate that caution should be taken when evaluating ^{86}Rb leakage (electrolyte loss) from certain plant tissues.

Effect of purified enzymes on cucumber protoplasts.—When isolated protoplasts from cucumber locular tissue were treated with 0.01% neutral red in 0.6 M sucrose at pH 7.3, the dye accumulated within the vacuoles and cytoplasm. The effects of the various purified enzymes on protoplast survival are illustrated in Table 3. Both purified phosphatidase C and protease caused the protoplasts to burst, whereas endo-PGTE did

TABLE 2. Cellular death (CD) and maceration (M) ratings of potato and cucumber disks treated with phosphatidase C (PTD), protease (PRT), and endopolygalacturonate *trans*-eliminase (endo-PGTE) from *Erwinia carotovora* (EC14)[a]

		Enzyme treatment[b]													
		Endo-PGTE		PTD		PRT		Endo-PGTE + PTD		Endo-PGTE + PRT		PRT + PTD		PRT + PTD + Endo-PGTE	
Tissue	Hour	CD	M	CD	M	CD	M	CD	M	CD	M	CD	M	CD	M
Potato	1	2	2	0	0	0	0	2	2	1	1	0	0	1	1
	2	3	3	0	0	0	0	3	3	2	2	0	0	3	3
	3	4	4	0	0	0	0	5	5	4	4	0	0	5	5
	4	5	5	0	0	0	0	5	5	5	5	0	0	5	5
Cucumber	1	5	5	0	0	0	0	5	5	5	5	0	0	5	5
	2	5	5	0	0	0	0	5	5	5	5	0	0	5	5
	3	5	5	1	0	1	0	5	5	5	5	2	0	5	5
	4	5	5	2	0	1	0	5	5	5	5	2	0	5	5

[a]Cellular death and maceration of 8×0.2 mm disks were measured in 1.0 ml of enzyme preparation buffered with 0.05 M Tris-HCl (pH 8.0) at 30 C. A value of 0 indicates no maceration or cellular death, whereas a value of 5 indicates complete maceration or cellular death determined by failure to retain neutral red (23). All experiments were repeated four times with autoclaved enzymes or buffer (0.05 M Tris-HCl at pH 8.0) as controls.

[b]Units of the enzyme activity when used separately or in combination were the same; e.g., protease (6.6 units), phosphatidase C (3.3 units), and endopolygalacturonate *trans*-eliminase (13.3 units).

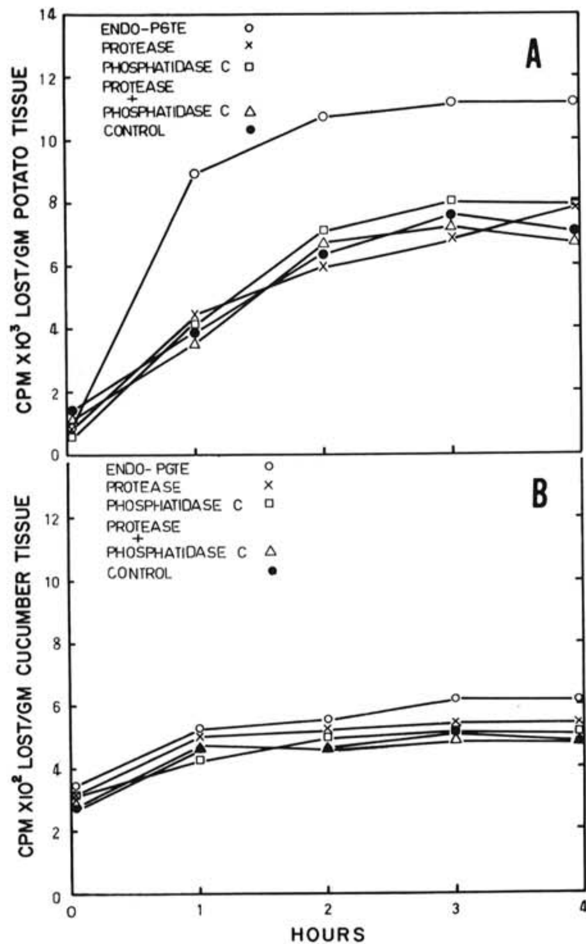

Fig. 3-A, B. Leakage of ^{86}Rb from (A) potato disks and (B) cucumber disks treated with purified endopolygalacturonate *trans*-eliminase, protease, phosphatidase C, and protease + phosphatidase C. Reaction mixtures contained 2.0 ml of enzyme, 2.0 ml of 0.05 M Tris-HCl buffer (pH 8.0) and eight disks. The control is represented by the leakage of ^{86}Rb from potato and cucumber disks when reacted with autoclaved enzyme-buffer mixtures. Enzyme activities were the same as those described in Table 2.

not. When phosphatidase C and protease were combined in a reacion mixture, there was a decrease in the percent survival of cucumber protoplasts compared to treatment with these enzymes used singly in a reaction mixture (50% and 76% survival after 1 h incubation for phosphatidase C and protease, respectively, compared to 36% when the enzymes were in combination). When the protoplasts were treated with either phosphatidase C or protease (Fig. 4), leakage of neutral red was observed many times before bursting was evident or at the moment just before bursting occurred. However, endo-PGTE-treated protoplasts always retained the dye.

DISCUSSION.—To understand the involvement of various enzymes produced by pathogens in disease development, highly purified enzyme systems must be used (16). In this study three enzymes—endo-PGTE, protease, and phosphatidase C—were selected for purification. Although endo-PGTE had been purified previously (11, 16), the techniques for phosphatidase C and protease purification from *E. carotovora* had not been reported. Purification of these enzymes was accomplished by a combination of ammonium sulfate fractionation, DEAE cellulose column chromatography, and isoelectric focusing which resulted in a high yield of the enzymes.

Two phosphatidase fractions were eluted from the DEAE cellulose column. Both phosphatidases were determined to be of the C-type. In previous investigations (15, 16) only one phospholipid-degrading enzyme from *E. carotovora* (EC14) had been found, which may have been a reflection of the medium used to grow the bacteria. In the previous works, phosphatidase was isolated from culture filtrates of *E. carotovora*-inoculated nutrient broth solutions, whereas in this study the enzyme was purified from culture filtrates of *E. carotovora*-inoculated bean hypocotyls. The fact that two phosphatidase C fractions were obtained in this study may suggest that two "isoenzymes" were induced and may be a reflection of the autoclaved bean substrate used. Several investigators have demonstrated the presence of multiple phosphatide-degrading enzyme systems of fungal and bacterial origin (8, 12, 17).

The fact that purified endo-PGTE, as well as endo-polygalacturonase, will kill plant cells is well established (11, 16, 23). Purified endo-PGTE is capable of inducing maceration, electrolyte loss, and cellular death (16). The question here was whether endo-PGTE causes ion

TABLE 3. Percent survival of cucumber protoplasts treated with phosphatidase C, protease, and endopolygalacturonate *trans*-eliminase (endo-PGTE) from *Erwinia carotovora* (EC14)

Enzyme treatment[a]	Percent survival of cucumber protoplasts after exposure times of 0 h	1 h	2 h	3 h	4 h
Control[b]	100	100	100	100	100
Phosphatidase C	100	50	32	15	6
Protease	100	76	53	27	22
Endo-PGTE	100	100	100	100	100
Phosphatidase C + Protease	100	36	24	10	3
Phosphatidase C + Endo-PGTE	100	58	40	20	9
Protease + Endo-PGTE	100	68	47	42	34
Phosphatidase C + Protease + Endo-PGTE	100	47	20	17	2

[a]Each reaction mixture contained either Ca^{2+} or Mg^{2+} as a cofactor; protease (3×10^{-4} M Ca^{2+}), phosphatidase C (3×10^{-4} M Mg^{2+}), and endo-PGTE (3×10^{-4} M Ca^{2+}). Units of enzyme activity are the same as those described in Table 2.

[b]Control represents either reaction mixtures containing autoclaved enzyme with their respective cofactors or buffer alone.

leakage and cellular death in the absence of the host cell wall. The suggestion that endo-PGTE could react directly with the cell membrane and/or protoplast is due to the observation that leakage (assayed with ^{86}Rb) occurs before maceration and cellular death of host tissue are detected (16). If endo-PGTE acts directly on the membrane surface of the protoplast or the protoplast itself, one would expect to observe leakage of the neutral

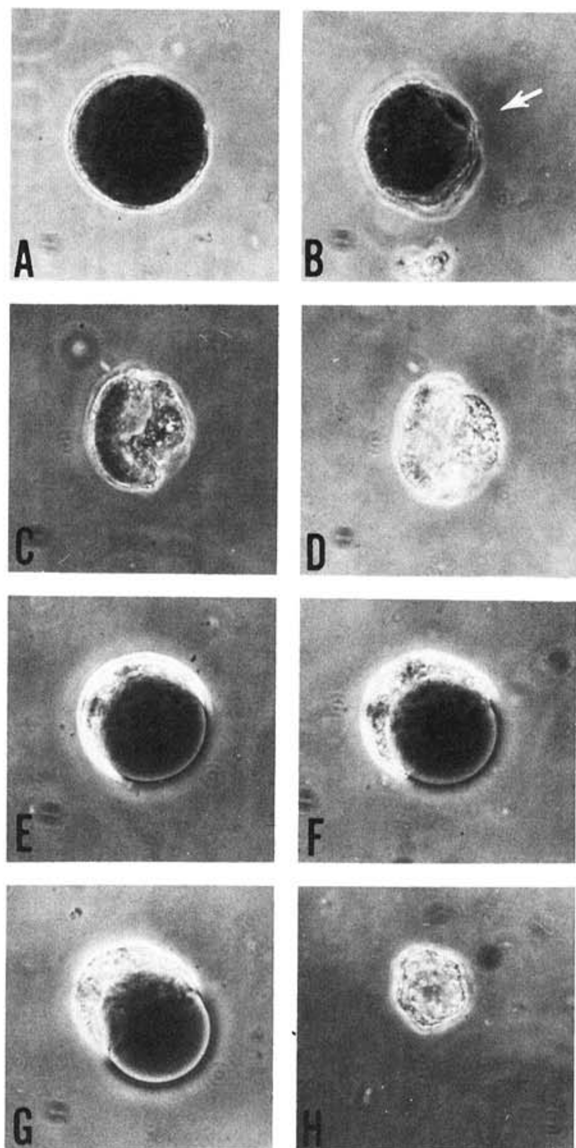

Fig. 4-(A to E). The sequential bursting of cucumber locular protoplasts after treatment with purified phosphatidase C and protease. A through D shows the condition of a single protoplast after treatment with phosphatidase C at 0, 3, 5, and 7 min, respectively. The arrow in (B) indicates the release of neutral red from the protoplast at the time of bursting. E through H shows the condition of a protoplast containing a large vacuole after treatment with protease at 0, 10, 45, and 60 min, respectively. The enzyme activities were the same as those described in Table 2. Under phase microscopy (× 257).

red dye from the protoplast or cell membrane disruption. Since endo-PGTE did not cause any observable changes in the protoplasts, any direct effects of it on the cell membrane or protoplasts in the absence of the cell wall appear questionable. Whether the contracted condition of the protoplasts (in 0.6 M sucrose) prevents or retards the action of the enzyme on receptor sites or substrates which may be present in the cell membrane or within the protoplasm, was not established.

Since phosphatidase C and protease are both produced in high amounts by *E. carotovora,* there are several questions of interest concerning these enzymes: Are they involved in pathogenesis? Do they cause permeability changes or chemical disruption of the cell membrane? Is maceration or degradation of the cell wall (by pectic enzymes) necessary before these enzymes can react with the cell membrane?

Phosphatidase C and protease were both capable of inducing some cellular death in intact cucumber tissue without maceration, but had no effect on potato tissue. Whether or not this demonstrates substrate differences between the two tissues was not determined. The bursting of cucumber protoplasts was striking, with phosphatidase C showing the greatest effect. In many instances, the leakage of neutral red occurred before bursting of the plasmolyzed protoplasts was evident, indicating a permeability change of the membrane. Studies with phospholipases from snake venom (7) and *Clostridium welchii* (2) indicated that these enzymes are quite toxic to animal tissue. This toxicity is believed to be associated with the destruction of phosphatides in biological membranes (21). The results reported here support the view of membrane modification (protein and phosphatide components) by phosphatidase C and protease. Phosphatidase C from *E. carotovora* also caused a decrease in oxygen uptake of isolated mitochondria from potato tissue (*unpublished*) which may again indicate membrane modification or a change in mitochondrial stability.

A possible interaction of proteins and cell membranes which may be responsible for cellular death was reported by Ruesink and Thimann (20). They tested a number of enzymes including proteinases and phosphatidase for their ability to burst isolated oat coleoptile protoplasts. No significant attack on the membrane was initiated by proteinases and phosphatidase. Ribonuclease was the only enzyme which induced the greatest lysis of the isolated protoplasts. This enzyme has an isoelectric point at 9.6, and they suggested that its bursting action may be due to its basic properties which may enable it to disrupt critical anionic sites on the membrane surface. In the present study purified phosphatidase C (pI = 7.5) and protease (pI = 8.3) produced by *E. carotovora* caused bursting of cucumber protoplasts, while purified endo-PGTE (pI = 9.4) did not. If ion imbalance is the important factor in the killing action of endo-PGTE, then one would have expected to see bursting of protoplast in the presence of this enzyme. However, one cannot rule out the possibility that plasmolysis (protoplasts in 0.6 M sucrose) stabilizes the ionic condition and therefore prevents the action of endo-PGTE (net positive charge) with the cell membrane (net negative charge on the outer surface). The fact that phosphatidase C and protease, which have lower

isoelectric points than endo-PGTE, caused bursting of cucumber protoplasts, suggests that protoplast lysis is due to the enzymatic action of these enzymes on the membrane.

The mechanism(s) of killing of host cells by purified endo-PGTE is still not established. At least in the cucumber system, cellular death can be induced with phosphatidases and proteases in the absence of pectic enzymes which induce tissue maceration. The demonstration that these enzymes are toxic to certain plant tissues suggests that they may be involved in disease development.

The implication of other phosphatidases (B-type) associated with abnormal or disease processes remain to be investigated in detail. Both *Sclerotium rolfsii* and *Botrytis cinerea* are able to produce phosphatidase B (25, 26). Perhaps, more detailed studies of this group of enzymes and of their association with plant pathogenesis may provide a better understanding of their toxic effects on host tissues.

LITERATURE CITED

1. ALBERSHEIM, P., and U. KILLIAS. 1963. Histochemical localization at the electron microscope level. Amer. J. Bot. 50:732-745.
2. ANSELL, G. B., and S. SPANNER. 1965. The action of phospholipase C on ethanolamine plasmologen (2-Acyl-1-alkenyl-glycerylphosphoryl-ethanolamine). Biochem. J. 97:375-379.
3. BATEMAN, D. F. 1963. The 'macerating enzyme' of Rhizoctonia solani. Phytopathology 53:1178-1186.
4. BATEMAN, D. F. 1972. The polygalacturonase complex produced by Sclerotium rolfsii. Physiol. Plant Pathol. 2:175-184.
5. BROWN, W. 1915. Studies in the physiology of parasitism. I. The action of Botrytis cinerea. Ann. Bot. 29:313-348.
6. BUTT, V. S., and H. BEEVERS. 1966. The plant lipids, p. 265-414. *In* F. C. Steward [ed.], Plant Physiology, Vol. IV-B. Academic Press, N.Y.
7. CONDREA, E., and A. DEVRIES. 1965. Venom phospholipase A: a review. Toxicon 2:261-273.
8. DOERY, H. M., B. J. MAGNUSSON, J. GULASEKHARAM, and J. E. PEARSON. 1965. The properties of phospholipase enzymes in staphylococcal toxins. J. Gen. Microbiol. 40:283-296.
9. FRIEDMAN, B. A. 1962. Physiological differences between a virulent and a weakly virulent radiation-induced strain of Erwinia carotovora. Phytopathology 52:328-332.
10. FUSHTEY, S. 1957. Studies in the physiology of parasitism. XXIV. Further studies on the killing of cells by fungal and bacterial extracts. Ann. Bot. (N.S.) 21:273-286.
11. GARIBALDI, A., and D. F. BATEMAN. 1971. Pectic enzymes produced by Erwinia chrysanthemi and their effects on plant tissue. Physiol. Plant Pathol. 1:25-40.
12. HAYAISHI, O., and KORNBERG. 1954. Metabolism of phospholipids by bacterial enzymes. J. Biol. Chem. 296:647-663.
13. 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.
14. LUMSDEN, R. D., and D. F. BATEMAN. 1968. Phosphatide-degrading enzymes associated with pathogenesis in Phaseolus vulgaris infected with Thielaviopsis basicola. Phytopathology 58:219-227.
15. MOUNT, M. S., and D. F. BATEMAN. 1969. Production of phosphatidase C by Erwinia carotovora. Phytopathology 59:401 (Abstr.).
16. MOUNT, M. S., D. F. BATEMAN, and H. G. BASHAM. 1970. Induction of electrolyte loss, tissue maceration, and cellular death of potato tissue by an endopolygalacturonate trans-eliminase. Phytopathology 60:924-931.
17. OI, S., and Y. SATOMURA. 1963. Purification and substrate specificates of phospholipase A and B of Sclerotinia fungus. Agric. Biol. Chem. 27:397-404.
18. 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.
19. RUESINK, A. W. 1971. The plasma membrane of Avena coleoptile protoplasts. Plant Physiol. 47:192-195.
20. RUESINK, A. W., and K. V. THIMANN. 1965. Protoplasts from the Avena coleoptile. Proc. Natl. Acad. Sci. USA 54:56-64.
21. SLEIN, M. W., and G. F. LOGAN, JR. 1965. Characterization of the phospholipase of Bacillus cereus and their effect on erythrocytes, bone, and kidney cells. J. Bacteriol. 90:69-81.
22. SPALDING, D. H. 1969. Toxic effect of macerating action of extracts of sweet potatoes rotted by Rhizopus stolonifer and its inhibition by ions. Phytopathology 59:685-692.
23. TRIBE, H. T. 1955. Studies in the physiology of parasitism. XIX. On the killing of plant cells by enzymes from Botrytis cinerea and Bacterium aroideae. Ann. Bot. (N.S.) 19:351-371.
24. TSENG, T. C., and D. F. BATEMAN. 1968. Production of phosphatidases by phytopathogens. Phytopathology 58:1437-1438.
25. TSENG, T. C., and D. F. BATEMAN. 1969. A phosphatidase produced by Sclerotium rolfsii. Phytopathology 59:359-363.
26. TSENG, T. C., S. L. LEE, and L. H. CHANG. 1970. An extracellular phosphatidase produced by Botrytis cinerea in vitro. Bot. Bull. Acad. Sin. (Taipei) 11:88-97.
27. VESTERBERG, O., T. WADSTROM, K. VESTERBERG, H. SVENSSON, and B. MALMGREN. 1967. Studies on extracellular proteins from Staphylococcus aureus. I. Separation and characterization of enzymes and toxins by isoelectric focusing. Biochim. Biophys. Acta 133:435-445.