Induction of Electrolyte Loss, Tissue Maceration, and Cellular Death of Potato Tissue by an Endopolygalacturonate Trans-Eliminase

M. S. Mount, D. F. Bateman, and H. Grant Basham

Former Post Doctoral Research Associate (present address, Department of Plant Pathology, University of Massachusetts, Amherst 01002), Professor, and Liberty Hyde Baily Research Assistant, respectively, Department of Plant Pathology, Cornell University, Ithaca, New York 14850.

Supported by Public Health Service Grant AI-04930 from the National Institute of Allergy and Infectious Diseases.

Accepted for publication 1 December 1969.

ABSTRACT

Extracts of *Erwinia carotovora*-infected potato tissues and filtrates of this pathogen cultured on nutrient broth supplemented with 0.1% sodium polypectate contained endopolygalacturonate *trans*-eliminase, proteinase, and phosphatidase C. These crude enzyme preparations contained a heat labile factor(s) that induced electrolyte loss, tissue maceration, and cellular death of potato tuber tissue discs. The endopolygalacturonate *trans*-eliminase was purified 295-fold from crude culture filtrate by ammonium sulfate fractionation, column chromatography on DEAE cellulose, and isoelectric focusing. The purified enzyme was free of proteinase, phosphatidase, and peroxidase activities, as well as enzymes which degrade araban, carboxymethyl cellulose, galactan, galactomannan, and xylan. This purified endopolygalacturonate *trans*-eliminase induced electrolyte loss, tissue maceration, and cellular death of potato tuber tissue. Electrolyte loss preceded maceration and cellular death. The results of this investigation suggest that a substrate for endopolygalacturonate *trans*-eliminase resides in the plant cell membrane or within the protoplast. Phytopathology 60:924-931.

Culture filtrates as well as extracts of plant tissues infected with a number of facultative plant pathogens contain a thermolabile, nondialyzable factor(s) that induces maceration and cellular death of plant tissues (9, 20, 34, 37). The early studies with *Sclerotinia libertiana, Bacillus carotovorus,* and *Botrytis cinerea,* by deBary (13), Jones (19), and Brown (9), respectively, clearly established the enzymatic nature of tissue maceration. These authors also described the killing action of enzyme preparations, but were hesitant to attribute maceration and killing to a common factor. Brown (9) attempted the separation of the activities responsible for maceration and cellular death in a systematic manner. Using an enzyme preparation from germinating spores of *B. cinerea,* he was unable to separate maceration and killing factors through differential inactivation by heat, mechanical agitation, or manipulation of H^+ concentration. Two hypotheses were proposed: (i) that cells were macerated and killed by the same substance (or group of substances); or (ii) that the cell walls were rendered permeable by the macerating substance to a separate, presumably colloidal, toxin.

Tribe (37) investigated this problem further, using enzymes produced by *B. cinerea* and *Bacterium aroideae.* Maceration and lethal activities paralleled each other in enzymes from both sources, and the degree of each activity appeared to be closely linked to the pectic enzyme concentration of the particular enzyme source. He was unable to separate maceration and lethal activities from each other or from the pectic enzymes, but he observed that plasmolysis of tissue resulted in a delay of the lethal response, while maceration was not appreciably affected. Further studies with these same experimental systems by Fushtey (17) confirmed Tribe's report concerning the increased resistance of plasmolyzed cells to the lethal action of these enzymes. Attempts to separate factors responsible for maceration and cellular death by differential precipitation with acetone failed. Also, it was demonstrated that the products released from the tissue during enzymatic maceration were not responsible for the lethal response (17). Tribe (37) offered the hypothesis "that the death of higher plant cells accompanying maceration by *Botrytis cinerea* and *Bacterium aroideae* enzymes is explainable by direct hydrolysis of the pectic materials present in them and accessible in unplasmolyzed living cells".

Kamal & Wood (20), working with enzymes from *Verticillium dahliae,* reported that the properties of the enzyme(s) responsible for maceration were similar to the enzyme(s) which caused viscosity loss of pectic substrates. They also reported that some separation of the maceration and lethal activities was obtained by heating enzyme solutions or by plasmolyzing the test tissue. In each case where a macerating enzyme has been purified and characterized, it has been shown to be an endopolygalacturonase or an endolyase which degrades the α-1,4 bonds in pectic substances (4, 6, 11, 12, 14).

There has been considerable reluctance on the part of investigators to consider that pectic enzymes per se could be responsible for the induction of lethal effects on plant tissue in spite of the numerous studies in which maceration and cellular death have been demonstrated to parallel each other. Tribe (37) suggested that lecithinases may act as toxic agents towards plant tissues. Recent studies in this laboratory have revealed that numerous facultative plant pathogens possess the ability to produce phosphatidases, and that these enzymes are readily obtained from *Sclerotium rolfsii*- and *Thielaviopsis basicola*-infected bean tissues (24, 39). Phosphatidases have been associated with the toxins of several animal pathogens (7, 15, 31). This group of enzymes as well as proteinases would appear to be likely enzymes for the induction of cellular death of plant tissues, since phospholipids and proteins are considered to be the primary components of plant cell

membranes (10). Pectic substances have not been considered as a constituent of cell membranes or protoplasm, although Albersheim & Killias (1) have reported that certain cytoplasmic particles of onion cells contain substances that are stained by alkaline hydroxylamine, and ferric chloride which has been used as a "specific" stain for pectic substances. Recent studies by Spalding (34) with the extracellular enzymes of *Rhizopus stolonifer* indicate that tissue maceration and cellular death of sweetpotato tissue are linked. Although the macerating enzyme used in this study was not extensively purified, it did not contain phosphatidase activity or appreciable proteinase. The macerating enzyme in the *R. stolonifer* system appears to be an endopolygalacturonase (33).

The objectives of this investigation were to determine (i) if tissue maceration and cellular death are separable; and (ii) to determine the enzyme system responsible for cellular death during tissue maceration. *Erwinia carotovora* and potato were selected as the host-pathogen combination for this study, since this pathogen is known to produce endopolygalacturonate *trans*-eliminase (35), proteinase (16), and phosphatidase (38); and the extracellular filtrates of this bacterium contain a heat labile, nondialyzable factor(s) which causes maceration and cellular death of potato tissue.

MATERIALS AND METHODS.—*Erwinia carotovora* (Jones) Holland, isolate EC_{14}, was used throughout this investigation. It was maintained on nutrient agar slants at 24 C; stock cultures were transferred at 2-week intervals. This isolate was highly virulent on potato tuber tissue (*Solanum tuberosum* L.). Infected host tissue serves as a good enzyme source, but since we wished to avoid enzymes of host origin, culture filtrates of *E. carotovora* grown in nutrient broth (Difco) supplemented with 0.1% sodium polypectate (Sunkist Growers, Ontario, Calif.) served as the main source of enzymes in this study, but some preliminary studies were made with enzymes from diseased tissues. *E. carotovora* was grown in 500-ml flasks containing 200 ml of the nutrient broth-sodium polypectate medium for 24 hr at 28 C on a shaker. Cells were removed by centrifugation (20,000 *g*) for 20 min at 4 C; the supernatant was used immediately as a crude enzyme source, or lyophilized and stored at −20 C.

Enzyme assays.—Since the phosphatidase produced by *E. carotovora* had not been identified (38), a number of assay procedures were employed to establish the type of phosphatidase produced by this pathogen. The 'cup plate' assay used as previously described (24) was employed for qualitative detection of phosphatidase activity. The assay medium contained 1% (w/v) refined soybean lecithin (Nutritional Biochemicals Corp.) in 0.1 M Tris [tris (hydroxymethyl) amino methane]-HCl buffer (pH 8.0), 1% agar, 0.1% (w/v) $MgSO_4$, and 0.01% Merthiolate. For the other assays, reaction mixtures contained 1.0 ml of a 1% refined soybean lecithin emulsion in 0.1 M Tris-HCl buffer (pH 8.0), 0.5 ml of enzyme, 0.5 ml of distilled water, and a drop of 0.05 M $CaCl_2$. Controls contained either water or autoclaved enzyme instead of active enzyme. Reaction mixtures were incubated for 1.0 hr at 30 C; enzyme activity was stopped by the addition of 0.1 ml of 5% bovine serum albumin and 0.9 ml of 20% trichloroacetic acid. Phosphatidase A and/or B activities were estimated by determining the loss in acyl-ester content of the substrate using the alkaline hydroxylamine procedure of Snyder & Stephens (32) as used by Lumsden & Bateman (24). The appearance of free choline in reaction mixtures was used as a criterion for phosphatidase D activity. Choline was determined by the method of Appleton et al. (3). Phosphatidase C was estimated by determining the release of acid-soluble phosphorus (phosphorylcholine) from soybean lecithin as described by Lumsden & Bateman (24).

Proteinase activity was estimated by two procedures. One was a 'cup plate' assay similar to the one described for phosphatidase, except that 1% gelatin was used as substrate and $MgSO_4$ was omitted. Proteinase was indicated by a clearing of a zone around the well (cup) to which 0.2 ml of enzyme had been added. The second procedure employed Congocoll (Calbiochem), a hide powder protein—Congo Red complex. Reaction mixtures containing 5 mg Congocoll, and 1.0 ml of enzyme in 0.05 M Tris-HCl buffer (pH 8.0) were incubated for 1 hr at 37 C. Each reaction mixture was made to 5.0 ml with distilled water and filtered. The Congo Red-protein complex released into solution from the Congocoll was read in a spectrophotometer at 495 nm. The absorbance at this wavelength was used as an index of relative proteinase activity.

Endopolygalacturonate *trans*-eliminase activity was measured by the periodate-thiobarbituric acid method (29), by the increase of reducing power of reaction mixtures (28), or by viscosity loss of sodium polypectate. Reaction mixtures assayed by the thiobarbituric acid and reducing group procedures contained 0.1 ml of 0.6% sodium polypectate in 0.05 M Tris-HCl buffer (pH 8.0) plus 10^{-4} M $CaCl_2$ and 0.1 ml enzyme. Incubation was for up to 1 hr at 30 C. Viscosity loss was determined with size 300 Fenske-Ostwald viscometers containing 4.0 ml 1.2% sodium polypectate buffered with 0.05 M Tris-HCl at pH 8.0, 1.0 ml of enzyme, and 1.0 ml of water. Measurements were made at 30 C (5). A unit of endopolygalacturonate *trans*-eliminase activity was defined as that amount of enzyme giving an increase in absorbance of 0.1 in 30 min at 548 nm in the thiobarbituric acid assay.

Purified preparations of endopolygalacturonate *trans*-eliminase were assayed for enzymes which attack araban, carboxymethyl cellulose, galactan, galactomannan, and xylan. Reaction mixtures contained 0.5 ml of 0.5% of the respective polysaccharide buffered with 0.05 M Tris-HCl at pH 8.0 and 0.5 ml of enzyme. Incubation was for 1 hr at 30 C; assays were made for increase in reducing groups on 0.2-ml samples (28). Peroxidase assays were made following the procedure described by Loebenstein & Linsey (22) in which pyrogallol is used as a substrate.

Fractionation of enzyme preparations.—Culture filtrates were brought to 40% saturation with ammonium sulfate, allowed to stand for 30 min at 15 C, centrifuged for 15 min at 20,000 *g*, and the precipitate was saved.

The supernatant was then brought to 60% saturation with ammonium sulfate, allowed to stand for 30 min, centrifuged, and the precipitate saved. This routine was repeated for ammonium sulfate saturations of 80 and 95%. The precipitate from each fraction was dissolved in water, dialyzed, and assayed for phosphatidase, proteinase, and endopolygalacturonate *trans*-eliminase activities.

The 60-80% ammonium sulfate fraction was applied to a 1.5 × 15 cm column of diethylaminoethyl cellulose (Cl^- form)(DEAE Cellulose) buffered with 0.05 M Tris-HCl buffer at pH 8.0. The column was eluted with 25 ml of buffer followed by a NaCl gradient in buffer until 0.4 M NaCl was reached. Five-ml fractions were collected and assayed for enzymatic activities.

The endopolygalacturonate *trans*-eliminase peak fractions from the DEAE cellulose column were combined and dialyzed for 24 hr against water. This enzyme fraction was subjected to isoelectric focusing in a LKB 8101 Ampholine electrofocusing apparatus equipped with a 110-ml column (LKB-Produkter AB, Bromma, Sweden) containing pH 3-10 or 7-10 Ampholine carriers (40). Electrofocusing was carried out at 21 C for 48 hr, at which time the pH gradient had formed and the current had stabilized (500 v, 0.8 ma). Five-ml fractions were collected at a flow rate of 110 ml/hr, and the pH of each fraction was measured. Each fraction was dialyzed against water at 21 C for 16 hr and then assayed for endopolygalacturonate *trans*-eliminase activity and protein content. This procedure was used to determine the isoelectric point of the enzyme as well as the final step in its purification.

The molecular wt of endopolygalacturonate *trans*-eliminase was estimated by sucrose density-gradient ultracentrifugation and by gel filtration. The sedimentation of the enzyme in a 5-20% sucrose gradient in 0.05 M Tris-HCl buffer (pH 8.0) was examined with a Beckman Model L 4 ultracentrifuge equipped with a SW-65 rotor. Density-gradient tubes were prepared and allowed to stand overnight at 4 C. The enzyme (0.2 ml) was layered on top of 4.5 ml density-gradients. Centrifugation was carried out for 11.5 hr at 60,000 rpm (240,000 *g*) at 8 C. Horseradish peroxidase (mol wt 40,000) (25) was used as a reference standard. After centrifugation, the contents of each tube were collected in 4-drop fractions; each fraction was assayed for endopolygalacturonate *trans*-eliminase activity. Peroxidase activity was measured by the method of Loebenstein & Linsey (22). The mol wt of the endopolygalacturonate *trans*-eliminase was estimated with this system according to the method of Martin & Ames (26). Gel filtration was accomplished with a 2.5 × 38 cm column of Sephadex G-75 equilibrated with 0.05 M Tris-HCl (pH 8.0) and 0.1 N KCl. The enzyme was applied to the surface of the column bed in a 1- to 2-ml volume containing 5-10 ml sucrose/ml, and 5-ml fractions were collected. Blue Dextran (mol wt 2,000,000), crystalline bovine serum albumin (mol wt 67,000), and cytochrome C (mol wt 12,400) were used as reference standards; estimates of the mol wt were made according to the procedure of Andrews (2).

Protein determination.—Protein measurements were made using the method of Lowry et al. (23). Crystalline bovine serum albumin was used as the reference protein.

Measurements of tissue maceration, permeability change, and cellular death.—The permeability change of potato tissue treated with enzymes was measured by determining the increase in conductivity of the bathing solution or by the loss of ^{86}Rb from tissue which had been allowed to accumulate $^{86}RbCl$ prior to enzyme treatment. Reaction mixtures for the conductivity studies contained enzyme, 10 potato discs (8 mm diam × 0.4 mm thick, about 0.5 g) in a 10-ml volume buffered with 0.01 M Tris-HCl at pH 8.0. Assays were made in 25-ml flasks placed on a wrist-action shaker (130 strokes/min) at 25 C. Controls consisted of tissue plus autoclaved enzyme or water. Conductivity measurements were made at time zero and at 1-hr intervals with a Model RD-15 solu bridge (International Instruments, Inc.). For the isotope leakage studies, 120 potato discs were placed in 10 ml of a solution containing 20 μc ^{86}Rb (1.3 μc ^{86}Rb/mg RbCl) buffered at pH 8.0 with 0.05 M Tris-HCl. The discs were stirred slowly for 4 hr at 25 C and then washed 6 times (100 ml each) with 0.025 M Tris-HCl buffer (pH 8.0). Fifteen discs (about 0.7 g) were put into test tubes containing 2.0 ml enzyme and 2.0 ml 0.05 M Tris-HCl buffer (pH 8.0). The controls contained autoclaved enzyme or distilled water instead of active enzyme. At time zero and indicated intervals thereafter, 0.1 ml of the bathing solution was removed and placed on a planchet. The planchets were dried overnight, and radioactivity was determined with a Nuclear-Chicago (Model 447) gas flow counter.

Cellular death was determined using the method described by Tribe (37). Potato discs were placed in enzyme buffered at pH 8.0 with 0.025 M Tris-HCl buffer. At various time intervals, discs were removed from the enzyme and transferred into a plasmolyzing solution containing Neutral Red and 1.0 ml of 0.05 M Tris-HCl buffer (pH 8.0). The discs were bathed in this solution for 20 min, then washed in 1 M KNO_3. Living cells were stained with the Neutral Red and the dye was retained after the 1 M KNO_3 wash. The dye readily leached from the dead cells during the wash. The quantity of living tissue in a potato disc was rated visually, using a 0-5 index. Discs showing no evidence of cellular death (dye loss) received a rating of 5; discs that were completely devoid of stained protoplasts after the wash received a rating of 0. Intermediate values were assigned to discs based upon the relative number of visibly stained protoplasts after the wash.

Maceration of potato discs after receiving the above treatments and being stained with Neutral Red was estimated and scored, using a 0-5 index. The tissue strength was estimated by teasing it apart with a spatula. Discs treated with autoclaved enzyme or water did not macerate, and received a score of 5, but those in which the coherence had been completely lost received a rating of 0. Intermediate values were assigned to discs exhibiting an intermediate stage of maceration.

Both the Neutral Red index and the maceration index are based on subjective observations, but with

practice a person can accurately distinguish differences within a rating of ± 0.5 on the 0-5 scale employed. In the experiments reported here, the treatments were randomized by one person and independently scored for cellular death and maceration by another. In order to minimize variation within a given experiment, potato discs were all prepared from the medulla tissue of a single potato tuber.

RESULTS.—Crude culture filtrates of *E. carotovora* as well as extracts of *E. carotovora*-infected potato contained phosphatidase, proteinase, and endopolygalacturonate *trans*-eliminase. These preparations also contained a heat labile factor(s) that induced loss of electrolytes, tissue maceration, and cellular death of potato tuber tissue discs. All studies were made at pH 8.0 since the enzymes under consideration were shown to be most active on the alkaline side of neutrality.

Phosphatidase activity.—Crude culture filtrates of *E. carotovora* gave a positive 'cup plate' assay for phosphatidase at pH 8.0, but not at 4.5 (38). This enzyme was contained in the protein precipitating between 40 and 60% ammonium sulfate saturation (Table 1). The phosphatidase was separated from endopolygalacturonate *trans*-eliminase and further purified by column chromatography on cellulose phosphate; the column was first eluted by a pH gradient (pH 6-8) and then by a salt gradient at pH 8.0 (0.05-0.4 M NaCl). Phosphatidase was eluted between 0.3 and 0.4 M NaCl (27). This enzyme was purified 51-fold from the crude filtrate, and contained 27.3 units of activity/mg protein, based on the release of acid-soluble phosphorus (24). The partly purified enzyme did not decrease the acyl-ester content of soybean lecithin or release free fatty acids from this substrate as determined by gas chromatography (39). Free choline was not released from lecithin, but the enzyme gave a positive 'cup plate' assay at pH 8.0 and released acid soluble phosphorus. It was concluded that *E. carotovora* produces phosphatidase C and releases phosphorylcholine from its substrate.

Proteinase activity.—*E. carotovora* filtrates added to 'cup plates' containing 1% gelatin produced a clear zone around the wells (cups) after 1 to 3 hr at 30 C. The crude enzyme also solubilized a Congo Red-hide powder complex (Congocoll). These two reactions were used to monitor proteinase activity. When crude enzyme preparations were fractionated with ammonium sulfate, the greatest amount of proteinase activity was found in the protein precipitate between 40 and 60% ammonium sulfate saturation (Table 1). This enzyme was easily separated from the endopolygalacturonate *trans*-eliminase by column chromatography on DEAE cellulose at pH 8.0 (Fig. 1). The endopolygalacturonate *trans*-eliminase was eluted with the void volume of the column, whereas the proteinase was eluted only after the addition of 0.4 M NaCl.

Endopolygalacturonate trans-eliminase activity.—The endopolygalacturonate *trans*-eliminase produced by *E. carotovora* exhibited activity on sodium polypectate or polygalacturonic acid at pH 8.0 but not at 4.5. Upon ammonium sulfate fractionation of crude enzyme, maximum endopolygalacturonate *trans*-eliminase activity was found in the precipitate obtained between 60 and 80% saturation with ammonium sulfate (Table 1). This fraction was applied to DEAE cellulose after dialysis, and the *trans*-eliminase was obtained in the void volume fractions (Fig. 1). The crude enzyme contained 20 units of endopolygalacturonate *trans*-eliminase activity/mg protein, and the combined active fractions (5-7) contained 119.5 units of activity/mg protein. Thus, a 6-fold purification was achieved by this two-step procedure.

Further purification of the endopolygalacturonate *trans*-eliminase was achieved by subjecting the active fractions from DEAE cellulose to electrofocusing. The initial studies were made using Ampholine carriers with a pH range of 3-10. The isoelectric point (pI) of the enzyme was estimated by this procedure to be pH 9.2.

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

$(NH_4)_2SO_4$ Fraction	Relative activity at pH 8.0		
	Phosphatidase[b]	Protease[c]	Endopolygalacturonate *trans*-eliminase[d]
% saturation			
40	0	50	17
60	100	100	56
80	0	33	100
95	0	0	47

[a] Culture was grown on nutrient broth supplemented with 0.1% sodium polypectate for 24 hr at 27 C.
[b] Assayed by the 'cup plate' method (24).
[c] Assayed by the Congocoll procedure.
[d] Assayed by the thiobarbituric acid procedure (29).

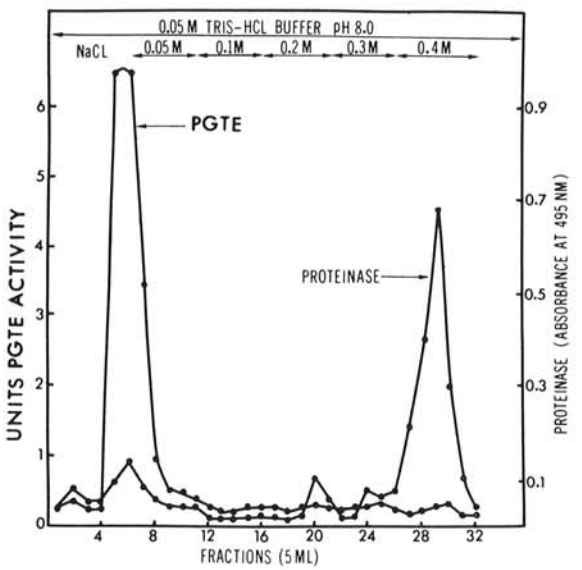

Fig. 1. Separation of endopolygalacturonate *trans*-eliminase (PGTE) from proteinase by column chromatography on DEAE cellulose. The enzyme source was the fraction obtained between 60 and 80% ammonium sulfate saturation of a crude culture filtrate of *Erwinia carotovora*. Endopolygalacturonate *trans*-eliminase activity is given in units per 0.1 ml.

Subsequent experiments were made using Ampholine carriers with a pH range of 7-10 (Fig. 2-A). The bulk of the protein applied to the electrofocusing column was separated from the endopolygalacturonate *trans*-eliminase. Combined fractions 3-7 from the electrofocusing column contained 5,913 units of endopolygalacturonate *trans*-eliminase activity/mg protein, which represents a 295-fold purification from the crude preparation. A summary of the purification scheme is presented in Fig. 3. This purified enzyme caused a 50% loss in viscosity of 1.0% sodium polypectate with less than 1% cleavage of the α-1,4 bonds.

The mol wt of this endopolygalacturonate *trans*-eliminase was estimated to be 31,000 by gel filtration

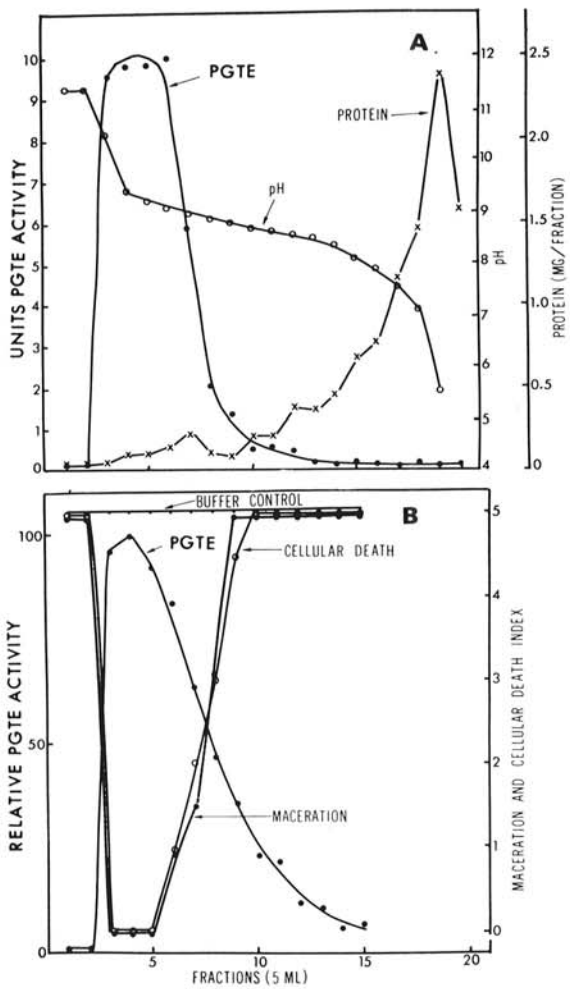

Fig. 2. **A)** Isoelectric focusing of 15 ml of dialyzed endopolygalacturonate *trans*-eliminase (PGTE) from DEAE cellulose (Fig. 1). Endopolygalacturonate *trans*-eliminase activity is given in units per 0.1 ml. The pI value for the enzyme is 9.2. **B)** Pattern of release of soluble unsaturated uronides (PGTE activity) from potato discs in reaction mixtures containing 10 potato discs, 0.5 ml of enzyme from fractions collected from the electrofocusing column, and 0.5 ml of 0.05 M Tris-HCl buffer (pH 8.0). Maceration and cellular death assays were made on potato discs in similar reaction mixtures after 6-hr incubation at 25 C.

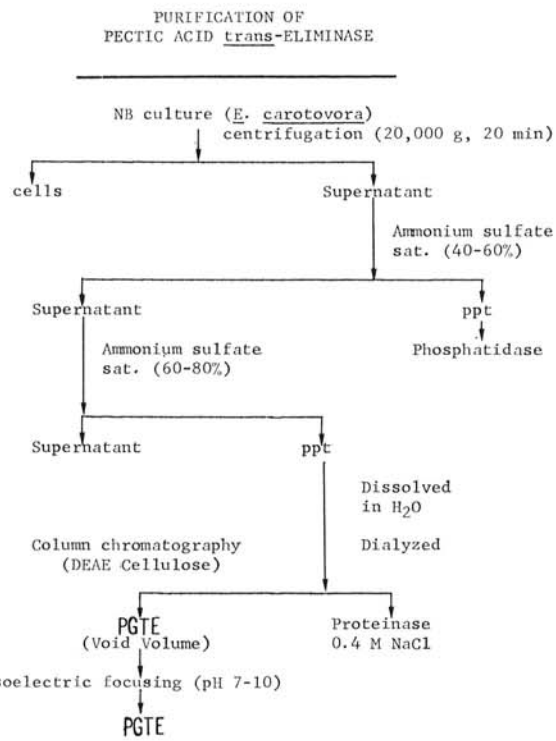

Fig. 3. Scheme for purification of the endopolygalacturonate *trans*-eliminase produced by *Erwinia carotovora*.

on Sephadex G-75. Crystalline bovine serum albumin (mol wt 67,000) and Cytochrome C (mol wt 12,400) were used as standards for this determination. Further estimates of the mol wt of this enzyme using sucrose density gradient centrifugation gave a value of 31,700 (Fig. 4-A).

Maceration, injury, and induction of cellular death by enzymes produced by E. carotovora.—The crude culture filtrate of *E. carotovora* contained a heat labile factor(s) responsible for leakage of electrolytes (Fig. 4-B), tissue maceration, and cellular death. Cellular death and maceration paralleled each other, but electrolyte loss preceded maceration and death.

Proteinase and phosphatidase failed to macerate or kill potato discs within a 2-hr period. The proteinase used for this study solubilized enough Congocoll to give an absorbance increase of 0.43 at 495 nm after 1-hr incubation at 37 C. The phosphatidase was free of endopolygalacturonate *trans*-eliminase, gave a positive 'cup plate' test for phosphatidase, and contained 1.5 units of phosphatidase activity/ml based on release of acid soluble phosphorus from soybean lecithin (24, 27).

Tissue maceration and cellular death occurred in all experiments in which the test enzyme solution contained endopolygalacturonate *trans*-eliminase. The action of the purified enzyme on sodium polypectate and induction of maceration and cellular death were directly correlated (Fig. 2-A, B). Also, the release of soluble unsaturated uronide products from potato discs by endopolygalacturonate *trans*-eliminase was directly

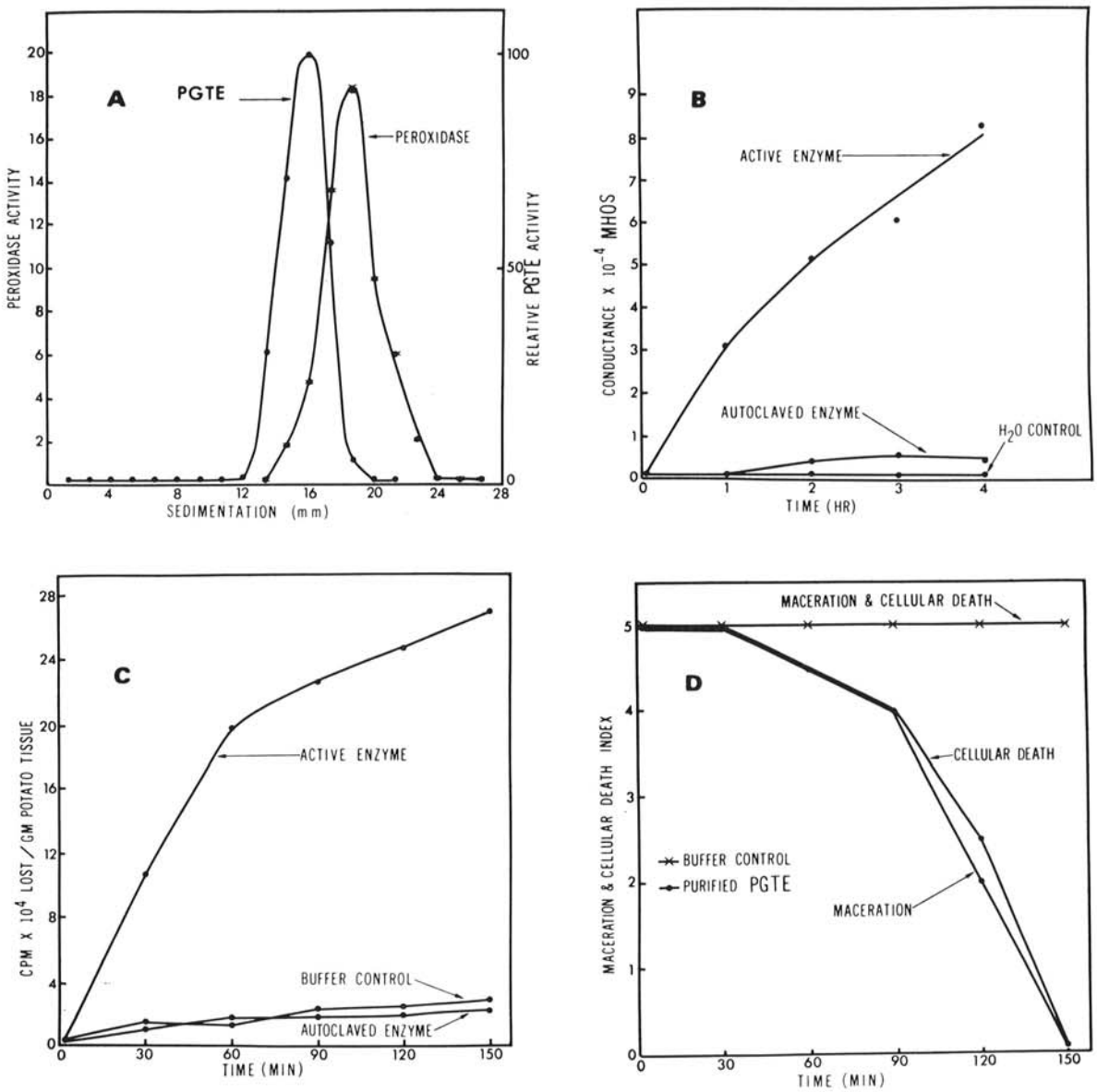

Fig. 4. A) Sedimentation of the endopolygalacturonate *trans*-eliminase (PGTE) of *Erwinia carotovora* and horseradish peroxidase in a 5-20% sucrose density-gradient when subjected to 240,000 *g* for 11.5 hr in a Beckman Model L 4 ultracentrifuge equipped with an SW-65 rotor. Enzyme assays were made on 4-drop (0.16 ml) fractions. **B)** Loss of electrolytes (increase in conductance of bathing solution) from 0.5 g of potato tissue discs in 10-ml reaction mixtures containing 1.0 ml of extract from *E. carotovora*-infected potato tissue and 0.01 M Tris-HCl buffer (pH 8.0). After 4 hr, no maceration was detectable and the cellular death index was 4. **C)** Leakage of ^{86}Rb from potato discs after treatment with purified endopolygalacturonate *trans*-eliminase. Reaction mixtures contained 2.0 ml (136.0 units) of endopolygalacturonate *trans*-eliminase, 2.0 ml 0.05 M Tris-HCl buffer (pH 8.0), and 15 potato discs (about 0.7 g). **D)** Maceration and cellular death of potato tissue discs treated with purified endopolygalacturonate *trans*-eliminase. Reaction mixtures contained 1.0 ml of enzyme (68.0 units of endopolygalacturonate *trans*-eliminase), 1.0 ml of 0.05 M Tris-HCl buffer (pH 8.0), and 15 potato discs (0.7 g).

correlated with tissue maceration and cellular death (Fig. 2-B).

The combined endopolygalacturonate *trans*-eliminase fractions (3-7) from the electrofocusing column were free of proteinase, phosphatidase, peroxidase, and enzymes which attack araban, carboxymethyl cellulose, galactan, galactomannan, and xylan at pH 8.0.

Experiments were made with the combined fractions (3-7) from an electrofocusing column which contained 68.0 units of endopolygalacturonate *trans*-eliminase activity/ml to determine the relationship between electrolyte leakage, maceration, and cellular death with the purified enzyme. Potato discs which had been allowed to accumulate $^{86}RbCl$ were treated with active enzyme,

autoclaved enzyme, and buffer alone. Isotope loss from tissue treated with the active enzyme was linear for the first 60 min, and amounted to 19.7×10^4 counts/min lost per g fresh wt of tissue; after 150 min, this figure had increased to 27.3×10^4 counts/min (Fig. 4-C). Isotope loss from the buffer and autoclaved enzyme controls over the 150-min experiment amounted to 2.6×10^4 and 2.5×10^4 counts/min per g fresh wt of tissue, respectively. Maceration and cellular death of potato discs were not detectable after 30 min of treatment, although both processes were detectable after 60 min. Both maceration and cellular death were complete after 150 min, and there was no indication of either maceration or cellular death in the buffer and autoclaved enzyme controls (Fig. 4-D). These experiments revealed that electrolyte loss precedes maceration and cellular death, and that the latter two processes parallel each other in the presence of highly purified endopolygalacturonate *trans*-eliminase.

DISCUSSION.—Increased loss of electrolytes by tissues invaded by plant pathogens has been observed by a number of investigators for a diverse group of diseases (18, 21, 36, 42). There is evidence that membrane permeability changes can be induced by low mol wt-toxins of pathogen origin as well as by enzymes (30, 37, 41). Thus, a number of mechanisms may be involved in permeability alterations during pathogenesis. The current study indicates that permeability change, tissue maceration, and cellular death of potato tissue can be caused by an endopolygalacturonate *trans*-eliminase produced by *E. carotovora.*

Proteinases and phosphatidases would appear to be likely candidates for induced permeability changes and induction of cellular death of plant cells, considering what is known concerning cell membrane composition (10); however, the results of the present investigation clearly associated permeability loss, maceration, and cellular death of potato tissue with endopolygalacturonate *trans*-eliminase activity. Though we did not obtain induction of cellular death with phosphatidase or proteinase produced by *E. carotovora* in this study, we do not wish, at this time, to conclude that these enzymes are not involved in the induction of cellular death during pathogenesis. Perhaps these latter enzymes, together or in combination with other factors, aid in killing plant cells. The fact that endopolygalacturonate *trans*-eliminase activity could not be separated from permeability loss, maceration, and cellular death raises several fundamental questions regarding the role of pectic enzymes in plant pathogenesis, as well as the possibility of the occurrence of galacturonic acid polymers within plant cell membranes or protoplasm. These particular points were raised by Tribe (37), and in view of the present investigation they should not be ignored.

The close association of the processes of maceration and cellular death reported in the literature (9, 17, 34, 37) has been supported by this study with a highly purified endopolygalacturonate *trans*-eliminase. The studies of Kamal & Wood (20) with pectic enzyme from *V. dahliae* in which they report some separation of maceration and cellular death by heating enzyme solutions and by plasmolyzing test tissue, we feel, can be explained on the basis of different kinetics for two processes caused by a common factor. The observation that plasmolyzed tissue is less rapidly killed in the presence of pectic enzymes is of particular interest (37), and suggests that (i) a substrate for the enzyme resides in the cell membrane and is less exposed when the membrane is in a contracted condition; or (ii) a substrate is within the protoplasm, and penetration of the contracted membrane by the enzyme is greatly retarded. The endopolygalacturonate *trans*-eliminase is a relatively small enzyme (mol wt about 31,000). Enzymes and macromolecules have been reported to penetrate plant cell membranes (8). There is a possibility that protoplasts are killed during maceration due to disruption of plasmodesmata, but if this is true, why should plasmolysis of tissue delay the killing response? Also, protoplast of higher plant cells can be kept alive up to 16 days free of cell walls (R. Schenk, *personal communication*). The cells in macerated tissue are killed prior to a physical disruption of tissue by mechanical means, i.e., cells die in the tissue discs after coherence of the tissue is lost even though the cells have not been physically separated from each other. In accordance with the hypothesis put forward by Tribe (37), we believe that maceration and cellular death of plant cells is explainable on the basis of direct degradation of pectic substances present and accessible in unplasmolyzed potato tuber tissue by pectic enzymes.

LITERATURE CITED

1. ALBERSHEIM, P., & U. KILLIAS. 1963. Histochemical localization at the electron microscope level. Amer. J. Bot. 50:732-745.
2. ANDREWS, P. 1964. Estimation of molecular weights of proteins by Sephadex gel-filtration. Biochem. J. 91:222-233.
3. APPLETON, H. D., B. N. LADU, B. B. LEVY, J. M. STEELE, & B. BRODIE. 1953. A chemical method for the determination of free choline in plasma. J. Biol. Chem. 205:803-813.
4. BATEMAN, D. F. 1963. The 'macerating enzyme' of *Rhizoctonia solani.* Phytopathology 53:1178-1186.
5. BATEMAN, D. F. 1966. Hydrolytic and *trans*-eliminative degradation of pectic substances by extracellular enzymes of *Fusarium solani* f. *phaseoli.* Phytopathology 56:238-244.
6. BATEMAN, D. F. 1968. The enzymatic maceration of plant tissue. Netherlands J. Pl. Pathol. 74 (Suppl. 1):67-80.
7. BERNHEIMER, A. W. 1968. Cytolytic toxins of bacterial origin. Science 159:847-851.
8. BRADFUTE, O. E., & A. D. MCLAREN. 1964. Entry of protein molecules into plant roots. Physiologia Plantarum 17:667-675.
9. BROWN, W. 1915. Studies in the physiology of parasitism. I. The action of *Botrytis cinerea.* Ann. Bot. 29:313-348.
10. BUTT, V. S., & H. BEEVERS. 1966. The plant lipids, p. 265-414. *In* F. C. Steward [ed.], Plant Physiology, Vol. IV-B. Academic Press, N.Y.
11. BYRDE, R. J. W., & A. H. FIELDING. 1968. Pectin methyl-*trans*-eliminase as the macerating factor of *Sclerotinia fructigena* and its significance in brown rot of apple. J. Gen. Microbiol. 52:287-297.
12. DEAN, M., & R. K. S. WOOD. 1967. Cell wall degradation by a pectate *trans*-eliminase. Nature (London) 214:408-410.

13. DeBary, A. 1886. Über einige Sclerotinien und Sclerotinienkrankheiten. Bot. Ztg. 44:377-474.
14. Demain, A. L., & H. J. Phaff. 1957. Recent advances in the enzymatic hydrolysis of pectic substances. Wallerstein Lab. Commun. 20:119-140.
15. Doery, H. M., B. J. Magnusson, J. Gulasekharam, & J. E. Pearson. 1965. The properties of phospholipase enzymes in staphylococcal toxins. J. Gen. Microbiol. 40:283-296.
16. Friedman, B. A. 1962. Physiological differences between a virulent and a weakly virulent radiation-induced strain of *Erwinia carotovora*. Phytopathology 52:328-332.
17. 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.
18. Hancock, J. G. 1968. Effect of infection by *Hypomyces solani* f. sp. *cucurbitae* on apparent free space, cell membrane permeability, and respiration of squash hypocotyls. Plant Physiol. 43:1666-1672.
19. Jones, L. R. 1909. The bacterial soft rots of certain vegetables. II. Pectinase, the cytolytic enzyme produced by *Bacillus carotovorus* and certain other soft rot organisms. Vt. Agr. Exp. Sta. Tech. Bull. 147: 281-360.
20. Kamal, M., & R. K. S. Wood. 1956. Pectic enzymes secreted by *Verticillium dahliae* and their role in the development of the wilt disease of cotton. Ann. Appl. Biol. 44:322-340.
21. Lai, M. T., A. R. Weinhold, & J. G. Hancock. 1968. Permeability changes in *Phaseolus aureus* associated with infection by *Rhizoctonia solani*. Phytopathology 58:240-245.
22. Loebenstein, G., & N. Linsey. 1961. Peroxidase activity in virus-infected sweet potatoes. Phytopathology 51:533-537.
23. Lowry, O. H., N. J. Rosebrough, A. L. Farr, & R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
24. Lumsden, R. D., & D. F. Bateman. 1968. Phosphatide-degrading enzymes associated with pathogenesis in *Phaseolus vulgaris* infected with *Thielaviopsis basicola*. Phytopathology 58:219-227.
25. Maehly, A. C. 1955. Plant peroxidase, p. 801-813. *In* S. P. Colowick, & N. O. Kaplan, [ed.], Methods in Enzymology, Vol. II. Academic Press, N.Y.
26. Martin, R. G., & B. N. Ames. 1961. A method for determining the sedimentation behavior of enzymes: application to protein mixtures. J. Biol. Chem. 236: 1372-1379.
27. Mount, M. S., & D. F. Bateman. 1969. Production of phosphatidase C by *Erwinia carotovora*. Phytopathology 59:401 (Abstr.).
28. Nelson, N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153:375-380.
29. Preiss, J., & 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.
30. Samaddar, K. R., & R. P. Scheffer. 1968. Effect of the specific toxin in *Helminthosporium victoriae* on host cell membranes. Plant Physiol. 43:21-28.
31. Slein, M. W., & G. F. Logan, Jr. 1965. Characterization of the phospholipases of *Bacillus cereus* and their effects on erythrocytes, bone, and kidney cells. J. Bacteriol. 90:69-81.
32. Snyder, F., & N. Stephens. 1959. A simplified spectrophotometric determination of ester groups in lipids. Biochim. Biophys. Acta 34:244-245.
33. Spalding, D. H. 1963. Production of pectinolytic and cellulolytic enzymes by *Rhizopus stolonifer*. Phytopathology 53:929-931.
34. 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.
35. Starr, M. P., & F. Moran. 1962. Eliminative split of pectic substances by phytopathogenic soft-rot bacteria. Science 135:920-921.
36. Thatcher, F. S. 1942. Further studies of osmotic and permeability relations in parasitism. Can. J. Res. (Sect. C) 20:283-311.
37. 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.
38. Tseng, T. C., & D. F. Bateman. 1968. Production of phosphatidases by phytopathogens. Phytopathology 58:1437-1438.
39. Tseng, T. C., & D. F. Bateman. 1969. A phosphatidase produced by *Sclerotium rolfsii*. Phytopathology 59: 359-363.
40. Vesterberg, O., T. Wadstrom, K. Vesterberg, H. Svensson, & 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.
41. Wheeler, H., & H. S. Black. 1963. Effects of *Helminthosporium victoriae* and victorin upon permeability. Amer. J. Bot. 50:686-693.
42. Williams, P. H., & N. T. Keen. 1967. Relation of cell permeability alterations to water congestion in cucumber angular leaf spot. Phytopathology 57:1378-1385.