

Killing of Plant Cells by Pectic Enzymes: the Lack of Direct Injurious Interaction Between Pectic Enzymes or Their Soluble Reaction Products and Plant Cells

H. G. Basham and D. F. Bateman

Former Liberty Hyde Bailey Research Assistant and Professor and Chairman, Department of Plant Pathology, Cornell University, Ithaca, New York 14850. Present address of the senior author: Department of Plant Pathology, North Carolina State University, Raleigh, North Carolina 27607.

This work was supported by Grant No. AI04930 from the National Institutes of Health. We thank H. W. Mussell for a gift of purified preparations of endopolygalacturonase and exopolygalacturonase from *Verticillium albo-atrum*. Accepted for publication 25 July 1974.

ABSTRACT

An isozyme of endopectate lyase (EC e_{11} , pI 9.4) produced by *Erwinia chrysanthemi* was purified by adsorption to CM Sephadex, DEAE cellulose "batch preparation", CM Sephadex column chromatography, and isoelectric focusing. Disc gel electrophoresis of purified EC e_{11} lyase yielded a single protein band that caused lytic degradation of sodium pectate, as well as maceration and cell death of potato and tobacco pith tissue. Cell injury in tissue treated with EC e_{11} lyase was characterized by a rapid loss of ions and water from cytoplasm (ca. 50% in 1 hour), indicating damage to the plasmalemma of cells. Tissue plasmolysed to the point of incipient plasmolysis in the presence of enzyme was protected from ion loss, but not from cell wall breakdown. Several endopectic enzymes differing in pH optima, isoelectric points, and mechanisms of substrate cleavage (lytic or hydrolytic), macerated and killed tissue. There was no evidence for a direct interaction between pectic enzyme and the plasmalemma. When isolated tobacco protoplasts were subjected to osmotic stretching in the presence of EC e_{11} lyase, protoplast (plasmalemma) injury was not enhanced.

When tissue or isolated cell walls were treated with EC e_{11} lyase, no soluble toxic products or by-products were detected in supernatants of the digests. Upon fractionation of the enzymatic digests of isolated cell walls or tissue, the fractions toxic to tissues contained residual EC e_{11} lyase activity. Beet tissue, resistant to wall breakdown and cell injury by EC e_{11} lyase, was not injured by enzyme digests of tobacco tissue. Hydrogen peroxide (100mM) did not cause electrolyte loss from potato tissue, nor did it accumulate in tissue treated with EC e_{11} lyase. Loss of electrolytes (cell injury) in tobacco and potato tissue paralleled the course of lytic degradation of plant cell walls. All enzymes which macerated tissue caused cell death, but an exopolygalacturonase from *Verticillium* caused no significant maceration or cell death in potato tissue. The results of this study are consistent with the hypothesis that membrane damage in cells treated with endopectic enzymes results from a loss in the ability of enzymatically degraded plant cell walls to support the limiting membrane of the cell.

Phytopathology 65:141-153

Additional key words: soft rot, membrane permeability, *Verticillium albo-atrum*, *Rhizopus stolonifer*, phytotoxin.

Most phytopathogenic microorganisms produce pectic depolymerases which degrade the α -1,4 linkages between galacturonsyl moieties in polymers of galacturonic acid. In many cases, these enzymes have been shown to cause extensive cell wall breakdown in infected host tissue (1, 4, 6, 7).

Early studies (8, 16) suggested that the enzymes responsible for maceration of plant tissue were able also to cause death of plant cells; the results of subsequent work with a variety of pectic enzymes which had been purified to high specific activity, are consistent with this view (13, 23, 24, 25, 41). In no case, however, was the pectic enzyme preparation shown to be homogeneous. Many different substances produced by phytopathogenic microorganisms cause increased permeability of plant cells similar to that induced by pectic enzymes. Thus, it is important to establish whether or not a pure pectic

enzyme can cause cell injury. Cell injury by pectic enzyme preparations is characterized by a loss of ability of cells to accumulate Neutral Red (a vital stain), a rapid loss of ions and water, and a loss of the ability of cells to plasmolyse in hypertonic solutions (8, 15, 18, 23, 43). If tissue is plasmolysed during treatment with pectic enzymes, cells do not lose the ability to accumulate Neutral Red, but tissue is macerated (12, 43). The mechanism by which pectic enzyme preparations cause damage to cell membranes is not known.

Mount et al. (23) suggested that pectic enzymes may damage plant cells by direct interactions between the host plasmalemma and pectic enzymes, and that sites of membrane-pectic enzyme interaction may be masked when the plasmalemma contracts during plasmolysis. Pectic enzymes do not cause lysis of isolated protoplasts from cucumber (44) or onion (30). Neuraminidase, an

enzyme which hydrolyses the α -ketosidic bond of the terminal N-acetylneuramic acid moieties of polysaccharides associated with animal membranes, adhered to the surface polysaccharides of retina cell membranes (22). Electron micrographs of onion root tissue stained with a variety of polysaccharide-specific stains indicate that plant protoplasts have a polysaccharide fraction directly associated with the plasmalemma surface (33, 34). If polysaccharide components of plant membranes contain a pectic substrate, binding of pectic enzyme to the plasmalemma might occur.

Ruesink (36, 37) has shown that a variety of protein molecules with high net-positive ionic charges can interact with the plasmalemma of isolated *Avena* protoplasts and cause lysis of the protoplasts. Most pectic enzymes which have been characterized have an isoelectric point higher than their pH optimum (5, 10, 13, 23, 24, 25). Thus these enzymes should have a positive ionic charge and might interact with the plasmalemma of plant cells.

The breakdown products which result from digestion of plant tissue by pectic enzymes have been tested for their ability to cause cell injury. When the supernatant fluid from pectic enzyme digests are boiled to eliminate residual pectic enzyme activity, the fluid causes no damage to potato tissue (12). If the residual wall material from such digests is washed free of pectic enzyme, the washed wall material does not injure plant tissue (9). These experiments, however, would not detect a thermolabile, soluble reaction product or by-product that might cause cell death, and there is reason to believe that such products might exist. For example, when cauliflower floret tissue is treated with a purified pectic lyase from *E. carotovora*, breakdown of the cauliflower cell wall by the pectic lyase releases and activates a wall-associated enzyme, glucose oxidase. The oxidase generates hydrogen peroxide, which is the limiting factor in ethylene production in the system (20, 21). A purified endopolygalacturonase from *V. albo-atrum* has been reported to initiate the production of hydrogen peroxide in cotton leaf tissue. Necrotic leaf symptoms generated by the polygalacturonase were identical to those found in *Verticillium*-infected cotton plants. Several oxidase-substrate combinations which yield hydrogen peroxide produced identical necrotic symptoms in cotton leaves, and catalase inhibited the production of necrotic symptoms in polygalacturonase-treated leaves (25). Hydrogen peroxide has been reported to cause increased permeability in beet cells (39). Thus it is possible that heat labile reaction products or by-products of pectic enzyme digestion of plant cell walls are responsible for the permeability increase in plant cells treated with pectic enzymes.

The objectives of this study were: (i) to determine if a pure pectic enzyme can cause injury to plant cells, and (ii) to investigate mechanisms whereby pectic enzyme preparations injure plant cells.

MATERIALS AND METHODS.—*Erwinia chrysanthemi* McFad. & Dim. (isolate EC₂₃), originally isolated from an unknown species of *Philodendron*, was obtained from R. S. Dickey (Department of Plant Pathology, Cornell University). The isolate was

maintained and culture filtrates were prepared by the procedures of Garibaldi and Bateman (13).

Rhizopus stolonifer Ehr. ex Fr., isolated from rotted sweet potato, was obtained from D. H. Spalding (Market Quality Research Division, ARS, USDA, Beltsville, Md.). The fungus was maintained on potato-dextrose agar slants. The culture was transferred every 6 weeks and stored at 24 C. *R. stolonifer* was grown in potato broth with 1% sodium polypectate (Sunkist Growers, Ontario, Calif.), with the pH of the medium adjusted to 6.0. Fernbach flasks containing 400 ml of sterile medium were seeded with mycelium and spores from an actively growing colony of the fungus. Cultures were incubated for 5 days at room temperature; the fungal mat was removed by filtration through four layers of cheese cloth. The culture filtrate (1,200 ml) was centrifuged (12,000 g) for 15 minutes at 4 C, then was dialyzed against water overnight at 4 C.

Enzyme assays.—Pectic hydrolyase activity was assayed by determining the increase in reducing sugar in reaction mixtures by the arsenomolybdate procedure (29). Reaction mixtures contained 0.2 ml of enzyme, 0.4 ml of 0.2% sodium polypectate, 0.25 ml of 200 mM buffer, and 0.15 ml of water. Reaction mixtures were incubated for 30 minutes at 30 C. Reducing groups were determined on the total reaction mixture or a fraction thereof. Data are expressed in μ moles of D-galacturonic acid equivalents. One unit of enzyme activity releases 1.0 μ mole of reducing groups in 1 minute.

Viscosity reduction of sodium polypectate solutions by enzymatic cleavage of the pectic substrates was also used as a measure of enzyme activity. Reaction mixtures contained 5.0 ml of 1.2% sodium polypectate in 100 mM phosphate buffer (pH 6.0) and 1.0 ml of enzyme solution. Viscosity was measured in Fenske-Ostwald viscometers (size 300) at 30 C (3).

Lytic cleavage of pectic substrates was determined by following the increase in absorbance of reaction mixtures at 230 nm (42). Reaction mixtures contained 1.2 ml of 0.2% sodium polypectate, 0.85 ml of 100 mM Tris hydroxymethyl aminomethane (Tris)-HCl buffer (pH 8.5), 0.75 ml of water, and 0.2 ml of enzyme solution. Data are expressed as μ moles of product formed, based on a molar extinction coefficient at 230 nm of 4,600 for the unsaturated product (28). One unit of enzyme activity is that amount which produces 1 μ mole of unsaturated uronide in 1 minute.

Lytic cleavage was also determined by the periodate-thiobarbituric acid (TBA) method (45). Reaction mixtures contained 0.1 ml of 0.2% sodium polypectate in 100mM Tris-HCl buffer (pH 8.5) and 0.1 ml of enzyme solution. The mixtures were incubated at 30 C. The μ moles of product produced were determined by using a standard curve prepared from lytically degraded sodium polypectate; the μ moles of bonds broken in the standards were determined by the arsenomolybdate procedure and measurement of absorbance at 230 nm.

Peroxidase activity was estimated by following the oxidation of guaiacol in the presence of enzyme. Reaction mixtures contained 2.0 ml of 20 mM guaiacol, 1.0 ml of 200 mM phosphate buffer (pH 7.0), and 0.2 ml of enzyme solution in both cuvetts of a Beckman Model DB spectrophotometer. At zero time, 0.1 ml of 0.3% H₂O₂ was added to the sample cuvet and the increase in

absorbance at 470 nm was determined at intervals. Activity is reported as absorbance change per minute in the early stages of the reaction while the rate of reaction was approximately linear. When more accurate quantitation of peroxidase activity was required, the *o*-dianisidine-HCl procedure was used (48). One unit of enzyme activity was that amount which degrades 1.0 μ mole of H₂O₂ in 1 minute.

Catalase activity was assayed using an oxygen electrode (Gilson Medical Electronics, Middleton, Wis.) to measure the production of oxygen from H₂O₂. Reaction mixtures contained 0.5 ml of 100 mM phosphate buffer (pH 7.0), 0.85 ml of water, 0.02 ml of 30% H₂O₂, and 0.2 ml of enzyme. Oxygen evolution was followed at 25 C. One unit of catalase activity is that amount which produces 1.0 μ mole of O₂ in 1 minute.

Enzyme purification.—Materials used for column chromatography were carboxymethyl (CM) cellulose (Whatman CM 22), diethylaminoethyl (DEAE) cellulose (Whatman DE 52 or DE 22), and CM Sephadex (Pharmacia Fine Chemical, CM Sephadex C50). All column materials were prepared according to manufacturers' directions and were equilibrated in columns until ionic equilibrium with the starting buffer (pH and conductivity) was obtained. Column materials were often used more than once; in such cases they were regenerated by washing in several changes of 1.0 M NaCl in the eluting buffer and then repacked and equilibrated with buffer in the column.

Isoelectric focusing was performed according to manufacturers' directions in a 110-ml Ampholine LKB 8101 electrofocusing apparatus (LKB-Produkter AB, Bromma, Sweden) containing pH 7-10 or 3-10 Ampholine Carrier Ampholytes (LKB).

Freshly prepared culture filtrate of *E. chrysanthemi* (3 liters) was dialysed against three 16-liter changes of water

for 18 hours and then made to 50 mM in Tris-HCl buffer (pH 8.5). This material was passed through a CM Sephadex C50 column (5 × 10 cm) that had been equilibrated with 50 mM Tris-HCl buffer (pH 9.5). The solution was allowed to pass through the column at ca. 3 ml/min. The column was then washed with 200 ml of the buffer and the enzyme was eluted from the column at a flow rate of 1.5 ml/minute with 250 ml of 200 mM NaCl in the same buffer. This elutant was diafiltered on an Amicon UM-10 membrane (Amicon, Lexington, Mass.) with the Tris-HCl buffer and concentrated to about 100 ml. The CM Sephadex concentrate was stirred for 30 minutes with 12 g of DEAE cellulose (DE-52) pre-equilibrated with 50 mM Tris-HCl buffer (pH 8.5). The DEAE cellulose was removed by vacuum filtration with glass fiber filter paper (Whatman GF/A) and washed two times with 50-ml portions of the buffer. The filtrate and DEAE cellulose washings (DEAE cellulose "batch preparation") were combined, split into three 65 ml batches, and stored at -20 C for future use.

The DEAE cellulose batch preparation was fractionated on a CM Sephadex column (2.5 × 21 cm) equilibrated with 50 mM Tris-HCl buffer (pH 8.5). Sixty-five ml of the DEAE cellulose fraction were passed through the column at a rate of 0.5 ml/minute. The column was washed with 100 ml of the buffer, and the enzyme was eluted with a linear salt gradient in which 300 ml of the Tris-HCl buffer was the starting solution and 300 ml of 400 mM NaCl in the same buffer was the final solution. Ten ml fractions were collected and assayed for pectic lyase activity by following the increase in absorbance of reaction mixtures at 230 nm. The endopectate lyase, designated fraction C₁, that elutes at approximately 80 mM NaCl was pooled and dialyzed overnight.

The C₁ fraction was electrofocused in a pH 7-10

TABLE 1. Purification of an endopectic acid lyase isozyme from *Erwinia chrysanthemi* (EC) and endopolygalacturonase from *Rhizopus stolonifer* (RS)

Fraction ^a	Protein (mg/ml)	Enzyme activity ^b (units/ml)	Specific activity (units/mg protein)
<i>E. chrysanthemi</i>			
culture filtrate	0.174	2.7	15.5
CM Sephadex concentration	0.358	50.0	139.7
DEAE cellulose batch prep.	0.110	18.1	165.0
CM Sephadex column, (C ₁)	0.046	14.2	306.9
electrofoc (ef)	<0.015 ^c	5.6	>300
<i>R. stolonifer</i>			
culture filtrate	0.319	0.02	0.06
60-90% (NH ₄) ₂ SO ₄	0.496	0.23	0.46
CM cellulose, (RS ₁)	0.066	0.06	0.96

^aSee methods section for preparation of the fractions.

^bEC fractions were assayed by the 230-nm absorbance assay; RS fractions were assayed by the arsenomolybdate assay.

^cThe protein concentration in this fraction is too low to determine accurately by the Lowry assay.

gradient of Ampholine ampholytes for 4 days at 400 V. The column was eluted at a flow rate of 0.5 ml/minute and 3-ml fractions collected. The pH of each fraction was determined and the fractions were assayed for endopectate lyase activity by the 230 nm absorbance assay. The peak of pectate lyase activity, eluted at pH 9.4,

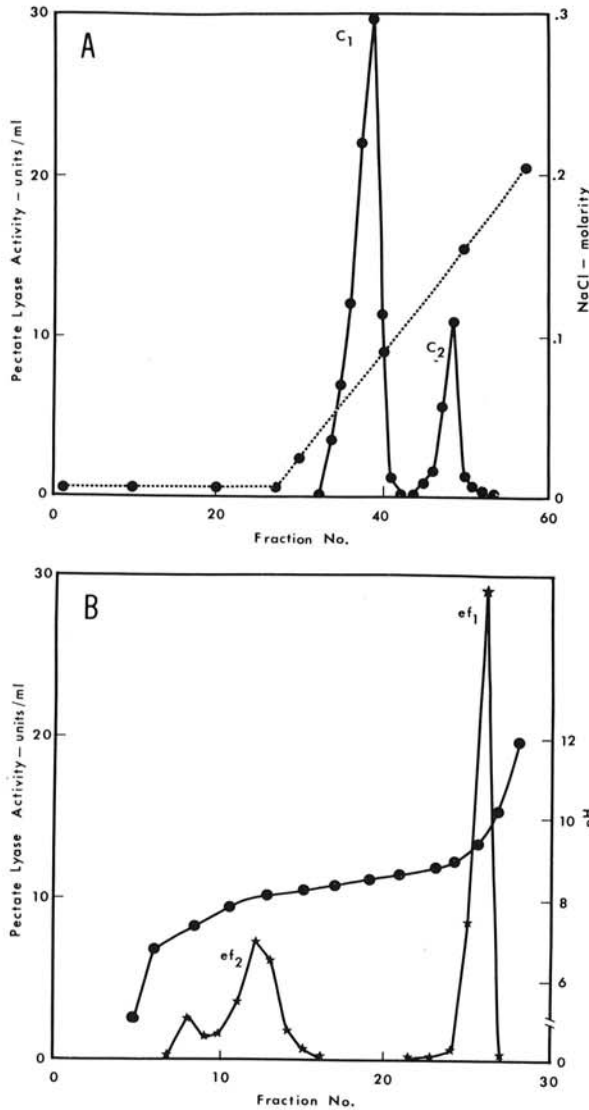


Fig. 1—(A, B). A) Elution profile of endopectate lyase from *Erwinia chrysanthemi* from a carboxymethyl (CM) Sephadex column. Sixty-five ml of enzyme from the diethylaminoethyl cellulose fraction were applied to the column (2.5×21 cm) at 4 C. The enzyme was eluted with 100 ml of 50 mM Tris-HCl buffer (pH 8.5), followed by a 600 ml linear concentration gradient of NaCl in Tris buffer (----). Enzyme activity (—) was measured by the 230 nm absorbance assay. B) Resolution of endopectate lyase isozymes from *Erwinia chrysanthemi* by electrofocusing. The C_1 fraction described in Fig. 1 (50 ml) was dialyzed and electrofocused using Ampholine ampholytes with a pH range of 7 - 10 at 4 C for 4 days. Three ml fractions were collected and pH values measured immediately (—●—); enzyme activity (—*—) was determined by the 230 nm absorbance assay.

was pooled and dialyzed overnight against 4 liters of 1.0 M NaCl to remove the ampholytes, and then against two 4-liter changes of water. This fraction is designated EC₁ lyase.

One liter of culture filtrate of *R. stolonifer* was brought to 60% saturation with ammonium sulfate, allowed to stand for 30 minutes at 20 C, and then centrifuged (20,000 g) for 15 minutes. The precipitate was discarded and the supernatant was brought to 90% saturation with ammonium sulfate, allowed to stand for 30 minutes at 20 C, and centrifuged (20,000 g) for 15 minutes. The precipitate was dissolved in 10 ml of water, dialyzed overnight against 50 mM phosphate buffer (pH 6.0), and fractionated at 4 C on a CM cellulose column (2.5×25 cm) equilibrated with the phosphate buffer. The column was eluted at a flow rate of 0.5 ml/minute and 5-ml fractions were collected. The endopolygalacturonase which eluted with the void volume of the column is designated RS.

Two purified polygalacturonases from *V. albo-atrum* were provided by H. W. Mussell (Boyce Thompson Inst., Yonkers, N. Y.). One enzyme, VA₁, is an endopolygalacturonase with a pH optimum of 6.0 and an isoelectric point (pI) of 9.3. The other enzyme, VA₂, is an exopolygalacturonase with a pH optimum of 5.2 and a pI of 6.5 (26).

Acrylamide gel electrophoresis.—Disc-gel electrophoresis was conducted in 0.6×4 cm gels of 7.5% acrylamide. With the following exceptions, the methods of Davis (11) for alkaline gels (pH 8.8) and of Riesfeld et al. (32) for acid gels (pH 4.3) were used. (i) Ammonium persulfate was used as a catalyst for polymerization of all gels. Separating gels contained 0.14% ammonium persulfate. Stacking gels contained 0.07% ammonium persulfate, and 0.05% N,N,N',N' tetramethylethylene diamine was substituted for riboflavin. (ii) Protein sample solutions were applied to the gels in 40% sucrose rather than in large-pore acrylamide gel. (iii) Neutral Red (0.001% in protein solutions) was used as a tracking dye in the acid gels. Protein solutions were made to 0.001% in tracking dye. Protein bands in gels were stained with 0.1% Amido Black in 7% acetic acid for 6 to 18 hours. Excess dye was removed electrophoretically in 7% acetic acid at 12 ma/gel.

Tissue maceration, cell death, permeability increase, and wall breakdown in tissue disks.—Potato (*Solanum tuberosum* L. 'Russett Burbank') tubers, beet (*Beta vulgaris* L.) roots, and cucumber (*Cucumis sativus* L.) fruits were purchased at a local market and stored at 4 C until used. Tobacco plants (*Nicotiana tabacum* L. 'Samsun NN') were grown in the greenhouse; the cultural procedures of Ross and Israel (35) were used. Tobacco leaf tissue was obtained from fully expanded leaves of 7- to 8-week-old plants and pith tissue from 9- to 11-week-old plants. For assays, disks were cut from medullary tissue of potato tuber, mesocarp of cucumber fruit, tobacco leaves, or tobacco pith.

Cell death was estimated by a modification of the Neutral Red assay of Tribe (43). Tissue disks (8mm diam \times 0.4 mm thick, ca. 0.04 g/disk) were treated with pectic enzyme. Duplicate tissue disks were removed at intervals after the addition of enzyme and placed in 1.0 ml of a solution containing 600 mM KNO₃, 1mM CaCl₂, 20

mM phosphate buffer (pH 7.5), and 0.01% Neutral Red. After 20 minutes, disks were rinsed in 3.0 ml of KNO_3 solution without the Neutral Red. Cell death was estimated visually, based on loss of ability of cells to accumulate Neutral Red. A cell-death index with a 0 to 5 linear scale was used. A rating of "0" indicates that all cells accumulated the dye, and a rating of "5" indicates that no cells accumulated the dye.

Maceration of enzyme-treated disks was estimated after the disks were stained with Neutral Red. The degree of cohesion between cells in the disks was determined by estimating the ease with which the disks could be pulled apart with spatulas. A maceration index with a 0 to 5 linear scale was used. A rating of "0" indicates that cellular cohesion was comparable to that in freshly cut disks and a rating of "5" indicates that there was no cohesion between cells in the disks.

Changes in the permeability of plant cells was measured by determining the loss of electrolytes or $^{86}\text{Rb}^+$ from tissue disks during treatment. For loss of electrolytes, disks (8 mm \times 1 mm, ca. 0.1 g/disk) were cut, washed for 1 minute in running tap water, and then rinsed with slow stirring at 4 C in 50 ml of 1 mM CaCl_2 per gram of disks (20 minutes/rinse for three rinses). Reaction mixtures contained rinsed disks in a 25-ml flask with 4 ml of enzyme in a standard reaction buffer containing 10 mM Tris-HCl buffer (pH 8.5) with 1 mM CaCl_2 , and antibiotics (50 $\mu\text{g}/\text{ml}$ penicillin G and 100 $\mu\text{g}/\text{ml}$ streptomycin sulfate). Reaction mixtures were shaken on a rotary shaker at ca. 120 cycles/minute at 25 ± 0.5 C. The conductance of the bathing solution was determined with a YSI conductivity apparatus (Yellow Springs Instrument Co., Yellow Springs, Ohio) at intervals after addition of enzyme. The total amount of electrolytes available in the tissue was determined for each reaction mixture by freezing the reaction mixtures in liquid nitrogen and then rapidly thawing in boiling water. The mixture was allowed to equilibrate for 30 minutes; then the conductance (μmhos) of the solution was determined. Loss of $^{86}\text{Rb}^+$ was determined using the method described by Mount et al. (23). Specific activity of $^{86}\text{Rb}^+$ was 0.11 $\mu\text{Ci}/\mu\text{mole}$. All uptake, rinse, and reaction solutions contained antibiotics and 18 mM RbCl . Total available $^{86}\text{Rb}^+$ in disks was determined by the freeze-thaw procedure used for electrolyte-loss measurements. Data are expressed as the percent of the total available electrolytes or $^{86}\text{Rb}^+$ lost from the tissue at intervals after the addition of enzyme. There were at least three replicates of all treatments in the assays of permeability alterations. Controls, consisting of autoclaved enzyme and/or water substituted for enzyme, were used for all treatments. Temperature for incubation of reaction mixtures was maintained at 25 ± 0.5 C.

Wall breakdown in potato disks was determined by measuring the release of unsaturated uronides from disks treated with pectate lyase. Tissue disks, reaction mixtures, and controls were identical to those in the electrolyte-loss assays. At intervals after the addition of enzyme, 0.1 to 0.6 ml of solution was removed from the reaction mixture and placed in a test tube with 0.05 ml of acetic acid. An equivalent amount of tissue (0.3 g/ml of solution removed) was removed at the same time to maintain a constant enzyme-to-tissue ratio. At the end of

the experiment, samples were heated in a boiling water bath for 1 minute; then the liquid was evaporated from the samples in an air stream at 40 C. The dried samples were taken up in 0.2 ml of water, and the unsaturated uronide content determined by the TBA method. Data are expressed as μmoles of unsaturated uronide released per gram of potato tissue.

Tobacco pith cell wall isolation.—Tobacco pith tissue (100 g) was ground in liquid nitrogen with mortar and pestle until all cells were broken. The ground tobacco tissue was homogenized in a blender for 5 minutes in 500 ml of 500 mM NaCl; temperature was kept below 10 C during homogenization. Cytoplasmic contaminants and ionically bound proteins were removed from the cell wall material by repeated wash cycles in detergent and salt solutions (31). All operations were carried out at 4 C. Walls were washed by stirring the wall material in five volumes of wash solution (10 min in salt solutions, 20 min in detergent solutions), centrifuging the suspension at 200 g for 1 minute, and decanting the wash solution from the wall material. The wash solution series consisted of 500 mM NaCl (one time), 1.0% Triton X-100 (two times), water (four to twelve times), 2.0 M NaCl (four to eight times), 5.0 M NaCl (two times), water (one time), 500 mM NaHCO_3 (two times), and water (four times). The initial water washes and the 2.0 M NaCl washes were repeated until no peroxidase activity was detected with the guaiacol

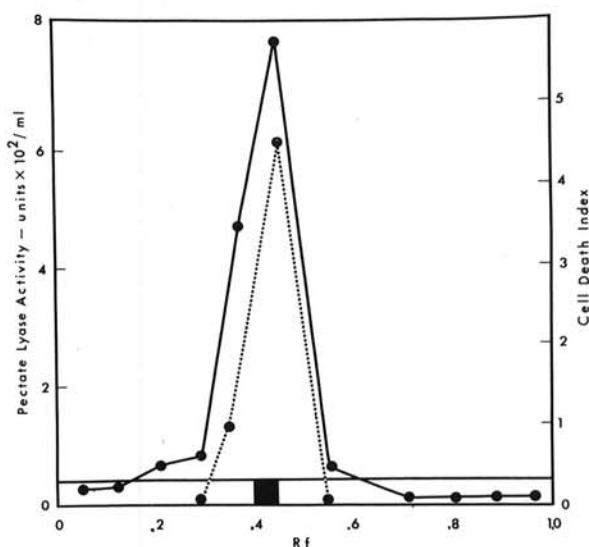


Fig. 2. Acrylamide gel electrophoresis of the purified endopectate lyase isozyme (EC ef_i) from *Erwinia chrysanthemi*. EC ef_i lyase (20 $\mu\text{g}/\text{gel}$) was electrophoresed on 7.5% polyacrylamide gels at pH 4.3. Duplicate gels were frozen and sliced transversely into 2 mm slices; then enzyme was eluted from each slice by steeping overnight at 4 C in 1.0 ml of 50 mM Tris-HCl buffer, pH 8.5. The cell death index (—) was determined for each fraction by the Neutral Red assay (0 = no effect, 5 = all cells killed) with potato disks incubated in 0.9 ml of a gel fraction of 2.5 hours. Enzyme activity (---) was determined by the TBA method. Protein was detected in an identical set of duplicate gels by staining with 0.1% Amido Black in 7% acetic acid. A stained gel is illustrated in the sketch on the abscissa of the figure.

assay on 0.5 ml of the wash solution after 15 minutes of incubation. Wall material was stored frozen for up to 1 week before use.

Protoplast isolation.—Protoplasts were isolated from mesophyll tissue of fully expanded tobacco leaves by the method of Goodman and Ross (14). Protoplasts were rinsed twice with 700 mM mannitol containing 0.1 mM CaCl_2 and stored up to 24 hours in the medium of Nagata and Takebe (27) to which 300 $\mu\text{g/ml}$ cephaloridine (Eli Lilly & Co., Indianapolis, Ind.) and 10 $\mu\text{g/ml}$ rimocidin (Pfizer, Inc., Groton, Conn.) were added to retard microbial growth.

Protein determination.—Protein was determined by the procedure of Lowry et al. (19). Crystalline bovine serum albumin was used as the reference protein.

RESULTS.—**Enzyme purification.**—An isozyme of endopectate lyase with an isoelectric point of 9.4 was purified from culture filtrates of *E. chrysanthemi* by the procedure of Garibaldi and Bateman (13). When this purified lyase was assayed by polyacrylamide gel electrophoresis (7.5% acrylamide, pH 4.3) for homogeneity, five protein components were detected.

A new purification procedure was developed to obtain a homogeneous isozyme preparation. The new purification process (Table 1) yielded, with 22-fold purification and 5-10% yield, an isozyme with a specific activity of ca. 300 units/mg protein. Protein content of the purified enzyme was too low to measure accurately by

the Lowry method, but occasional preparations contained up to 60 μg protein/ml. The specific activity of different preparations ranged from 260 to 320 units/mg protein. Enzyme yield in the purification process was difficult to determine since other isozymes of pectate lyase were removed at steps during purification. When culture filtrate was passed through a CM Sephadex column, some pectic acid lyase activity was not retained by the column and was discarded. The fraction retained was eluted with 400 mM NaCl in 50 mM Tris-HCl (pH 8.5). This fraction was stirred with DEAE cellulose previously equilibrated with the Tris-HCl buffer. Material adsorbed by the DEAE cellulose was discarded; the filtrate (supernatant) containing the desired enzyme fraction was layered on a CM Sephadex column and eluted with a NaCl gradient in the Tris-HCl buffer (Fig. 1-A). The lyase fraction (designated C_2) which eluted at ca. 140 mM NaCl was discarded. The fraction (designated C_1) which eluted at ca. 80 mM NaCl was dialysed and subjected to isoelectric focusing in Ampholine ampholytes with a pH range of 7 to 10 (Fig. 1-B). The C_1 fraction was resolved into two peaks of lyase activity. The isozyme eluting at pH 9.4 was designated EC ef_1 lyase. The fraction eluting at pH 7.9 was designated EC ef_2 lyase.

The EC ef_1 lyase had a pH optimum of 9.0 and an isoelectric point (pI) of 9.4. This isozyme was stable for at least 3 months when stored at -20°C ; it exhibited a distinct preference for nonmethylsterified pectic substrates. Relative activities of this isozyme on various substrates were: polygalacturonic acid, 1.00; sodium polypectate, 0.75; and pectin N.F., 0.11. This enzyme fraction yielded a single protein band when subjected to electrophoresis in acrylamide gels containing 7.5 or 12% acrylamide at pH 3.5 or 4.3. As expected, no protein bands were detected in alkaline gels since the pI of the enzyme was 9.4.

The purification of endopolygalacturonase from *R. stolonifer* (Table 1) yielded, with 16-fold purification, a partially purified enzyme with a specific activity of 0.96 units/mg protein. This polygalacturonase had a pH optimum of 5.5 when incubated with sodium polypectate in 50 mM citrate-phosphate buffer. No unsaturated uronides were released at pH 5.5 or 8.0 as determined by increase in absorbance of reaction mixtures at 230 nm. This enzyme hydrolysed sodium polypectate in a random (endo) manner; a 50% reduction in the viscosity of this substrate occurred after hydrolysis of only 0.2% of the available glycosidic bonds. Isoelectric focusing of this partially purified enzyme in a pH 3-10 gradient revealed a single peak of polygalacturonase activity with a pI of 5.5.

Injury to plant tissue by the EC ef_1 lyase isozyme.—When EC ef_1 lyase was electrophoresed on 7.5% acrylamide gels (pH 4.3), a single protein band was evident. Enzyme activity in fractions eluted from unstained sister gels was measured for lytic cleavage of sodium polypectate with the TBA method and injury to potato disks by the Neutral Red assay. Maximum lyase activity and maximum tissue injury occurred in the fractions coincident with the protein band in stained gels (Fig. 2). Maceration of potato tissue disks occurred only in fractions containing lyase activity.

Electrolyte loss from potato disks treated with EC ef_1

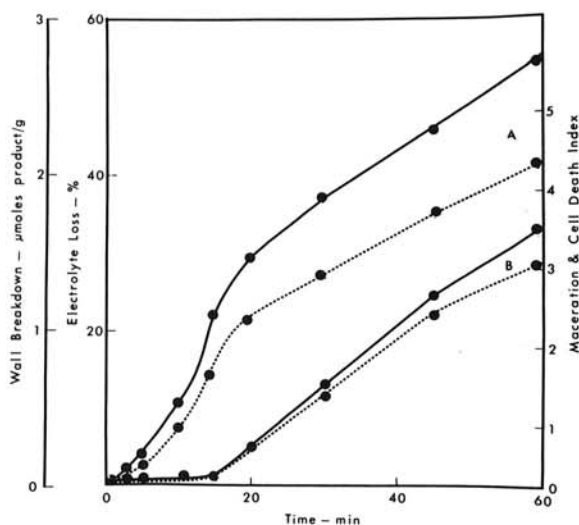


Fig. 3. Electrolyte loss, wall breakdown, cell death, and maceration in potato disks treated with endopectate lyase (EC ef_1) from *Erwinia chrysanthemi*. Potato disks (0.3 g/ml) were incubated with EC ef_1 lyase (0.35 units/ml). Measurements were made at intervals after the addition of enzyme. **A)** Wall breakdown (----) as μmoles of unsaturated uronides released from enzyme-treated tissue, and electrolyte loss (—) as % of electrolytes released from enzyme-treated tissue. In tissue disks treated with heat-inactivated enzyme, there was no wall breakdown and electrolyte loss was less than 2.5%/hr. **B)** Maceration index (----) and cell death index (—) in enzyme-treated potato disks (0 = no effect, 5 = complete maceration or cell death). In potato disks treated with heat-inactivated enzyme, maceration and cell death indices were both 0 at the end of the experiment.

lyase was detected within 3 minutes; one-half of the available electrolytes in enzyme-treated tissue were lost within 1 hour. Wall degradation during enzyme treatment paralleled electrolyte loss (Fig. 3-A). Increased conductance in reaction mixtures was not due to the release of ions from the cell wall during enzymatic degradation, since the total available electrolytes after membrane disruption at the end of the experiment was the same in reaction mixtures containing active and heat-inactivated enzyme (ca. 1,800 μ mhos with 0.3 g tissue/ml).

If injury to potato disks by EC ef_1 lyase was measured by the Neutral Red assay, no damage was observed until 15 minutes after the addition of enzyme; the extent of cell death in tissue (70% of cells killed in 1 hour) was comparable to the percent of electrolytes lost in the same period (60%). Tissue maceration paralleled the course of cell death (Fig. 3-B). Electrolyte loss and cell death were complete in such reaction mixtures within 1.5 to 2.0 hours.

Electrolyte loss from tobacco pith tissue paralleled the solubilization of unsaturated uronides in tissue treated with EC ef_1 lyase (Fig. 4-A). Increased electrolyte loss was detected within 1 minute after the addition of enzyme; electrolyte loss from tobacco pith tissue was complete within 2 hours. Electrolyte loss in tobacco pith tissue treated with heat-inactivated enzyme was substantial, but similar rates of electrolyte loss were observed from tissue treated with buffer alone.

Tobacco leaf disks lost about 25% of their electrolytes and 25% of their fresh weight within 30 minutes (Fig. 4-B) after exposure to the DEAE cellulose batch prep lyase fraction (Table 1) from *E. chrysanthemi*. Loss of fresh weight (water) roughly paralleled the course of electrolyte loss from this tissue.

Potato disks (0.12 g/ml) were plasmolyzed for 15 minutes in standard reaction buffer with 700 mM mannitol or sucrose. The plasmolyzed disks were treated with EC ef_1 lyase (0.75 units/ml) in standard buffer containing 700 mM mannitol or sucrose. Cell death was determined with the Neutral Red procedure. Cell death was not detected in plasmolyzed tissue at 30 or 60 minutes after the addition of lyase. In controls which were not plasmolyzed, cell death indices were 2.0 (30 minutes) and 3.5 (60 minutes) for enzyme-treated tissue. Maceration was only slightly inhibited by plasmolysis in sucrose. Maceration indices were 2.5 (30 minutes) and 3.5 (60 minutes) for disks plasmolyzed in sucrose and 2.5 (30 minutes) and 4.0 (60 minutes) for the unplasmolyzed controls. The wall breakdown rate in plasmolyzed potato disks treated with EC ef_1 lyase was not measured because both sucrose and mannitol interfered with the TBA assay. Lyase activity in 700 mM sucrose was inhibited by 30% when enzyme activity was measured by the 230-nm absorbance assay. Mannitol did not affect enzyme activity.

In order to determine the point of incipient plasmolysis of potato tissue, thin sections of potato were incubated for 30 minutes in solutions containing 50 to 400 mM sucrose, 10 mM Tris-HCl buffer (pH 8.5), 1.0 mM $CaCl_2$, and 0.01% Neutral Red. The percentage of cells plasmolyzed was determined with the use of a light microscope. Plasmolysis was first evident in 150 mM sucrose, and was complete in 300 mM sucrose. The point

of incipient plasmolysis (50% of cells plasmolyzed) occurred in solutions containing 200 to 250 mM sucrose.

To determine the degree of plasmolysis required for inhibition of cellular injury in potato tissue treated with

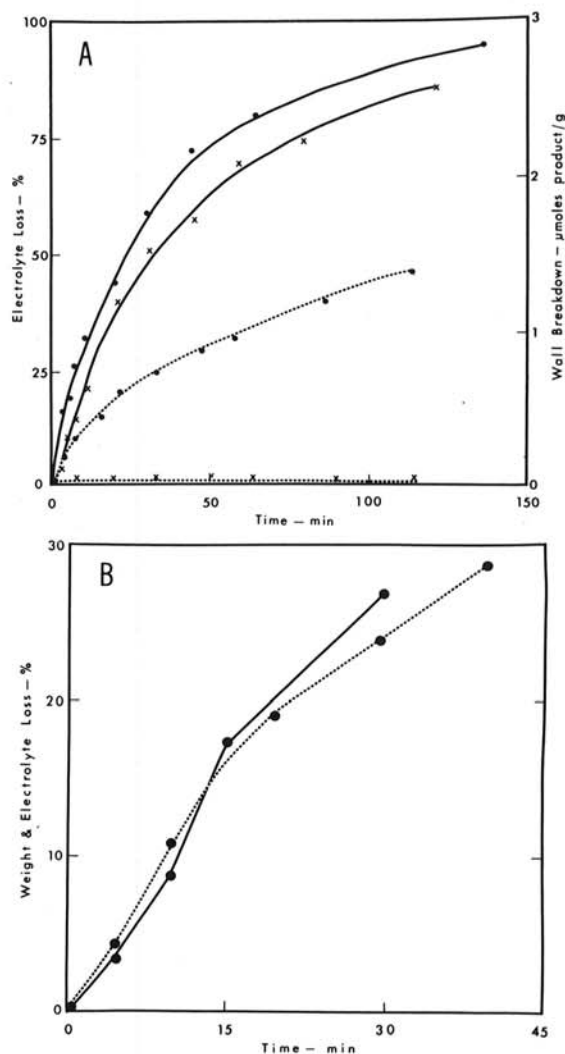


Fig. 4-(A,B). A) Electrolyte loss and wall breakdown in tobacco pith tissue treated with endopectate lyase (EC ef_1) from *Erwinia chrysanthemi*. Tobacco pith disks (0.17 g/ml) were incubated with EC ef_1 lyase (0.35 units/ml). Wall breakdown (X), as μ moles of unsaturated uronide released from tissue, and electrolyte loss, as % of electrolytes released from tissue (●), were measured at intervals after the addition of enzyme (—) or heat-inactivated enzyme (•••). B) Electrolyte loss and loss of fresh weight in tobacco leaf tissue disks treated with endopectate lyase (EC DEAE cellulose batch prep.) from *Erwinia chrysanthemi*. Tobacco leaf disks (0.03 g/ml) were incubated with lyase (0.075 units/ml) and, at intervals after the addition of enzyme, 6 disks and 2 ml of lyase solution were removed from each reaction mixture. Weight loss (•••) was determined by blotting and weighing the disks. Weight loss is expressed as a % of the initial weight. No measurements were made after maceration of disks became apparent. Electrolyte loss (—) was measured as % of total electrolytes released from the tissue. Electrolyte and water loss in disks treated with heat-inactivated lyase was less than 1% in 30 minutes.

EC ef_1 lyase, potato disks were allowed to accumulate $^{86}\text{Rb}^+$ at different sucrose concentrations, and then were treated at the same sucrose concentrations with EC ef_1 lyase (Fig. 5). The $^{86}\text{Rb}^+$ loss rate for lyase-treated tissue at or above the point of incipient plasmolysis was ca. 10% of that in unplasmolyzed tissue (with rates adjusted for leakage from controls). Loss of $^{86}\text{Rb}^+$ from plasmolyzed controls containing no enzyme was comparable to that in heat-inactivated enzyme controls.

Effect of enzyme characteristics on the ability of pectic enzymes to cause cell death.—Pectic enzymes with a variety of different specific characteristics were assayed for their ability to cause maceration and death of potato tissue (Table 2). The pH optimum, isoelectric point, net charge, and the mechanism of glycosidic bond cleavage (lytic or hydrolytic) by pectic enzymes did not affect their ability to cause maceration or cell death. Exopolygalacturonase from *Verticillium* caused little maceration and no cell death in potato disks during a 4-hour incubation period. When the concentration of this enzyme was doubled in reaction mixtures containing cucumber tissue, maceration and cell death were detected after a 3-hour incubation period. The maceration index was 2.5 and the cell death index was 1.0.

Effect of EC c_1 lyase on isolated tobacco leaf protoplasts.—The ability of EC c_1 lyase (Table 1) to kill

isolated tobacco leaf protoplasts was examined by incubating 0.1 ml of protoplasts (ca. 6×10^4 protoplasts/ml) with 1.8 units of lyase in 0.9 ml of 50 mM Tris-HCl buffer (pH 8.5) containing 700 mM mannitol. At intervals after the addition of enzyme, four 10- μ liter samples were withdrawn from each reaction mixture, and the percentage of living protoplasts was determined with the use of a phase-contrast microscope. Globular protoplasts having organized cytoplasm with chloroplasts arrayed adjacent to the plasmalemma were rated "living"; protoplasts with irregular shapes and/or disorganized cytoplasm were rated "dead". Enzyme-treated protoplasts remained alive after 90 minutes of incubation. Intact tobacco leaf disks in the absence of osmoticum lost 85% of their electrolytes in the same time period in reaction mixtures with similar enzyme concentration.

Since the contracted state of the plasmalemma in isolated protoplasts might alter interactions between the pectic lyase and the plasmalemma, protoplasts were subjected to osmotic stretching during enzyme treatment (Table 3). The percent of protoplasts killed at the different osmotic concentrations was similar in the presence and absence of active enzyme. When the concentration of osmoticum was decreased below 400 mM in reaction mixtures, the rate of protoplast death increased in the presence or absence of enzyme. Osmotic stretching did not render protoplasts susceptible to injury by the endopectate lyase.

Effect on tissue of breakdown products from pectic enzyme digests of plant tissue.—Peroxidase and catalase associated with plant tissue were solubilized when tissue or isolated walls were treated with pectic enzymes. Peroxidase activity associated with isolated tobacco cell walls was measured by substituting 0.2 ml of tobacco cell wall suspension for peroxidase in the guaiacol assay. No peroxidase activity was associated with cell walls autoclaved for 10 minutes at 121 C. Peroxidase activity in wall suspensions was linearly related to the amount of wall present in reaction mixtures over a range of 2 to 30 mg dry weight of wall. Dry weight of cell wall per ml in suspensions was determined turbidimetrically at 540 nm by using a standard curve.

Isolated tobacco cell walls (500 mg) were incubated for 2 hours with 1.5 units of EC ef_1 lyase in 10 ml of 50 mM Tris-HCl buffer (pH 8.5). The peroxidase activity in the wall preparation prior to enzyme treatment was 8.2 absorbance units/minute/g dry weight and the catalase activity was 1.0 units/g. Upon digestion with lyase, 60% of the wall was solubilized; the peroxidase and catalase activities per gram of residual cell wall were 6.2 absorbance units/g and 0.32 units/g, respectively. The supernatant contained peroxidase activity of 1.4 absorbance units/minute/g of solubilized wall and a catalase activity of 0.22 units/g of solubilized wall. No peroxidase was solubilized by treatment of walls with heat-inactivated enzyme.

An effort was made to determine if any of the enzymes removed from tobacco wall during lytic degradation were phytotoxic. Supernatant from the wall digest (8 ml) was electrofocused in a pH 3-10 Ampholine ampholyte gradient and 5-ml fractions were collected, dialyzed, and assayed for lyase activity, peroxidase activity, and the

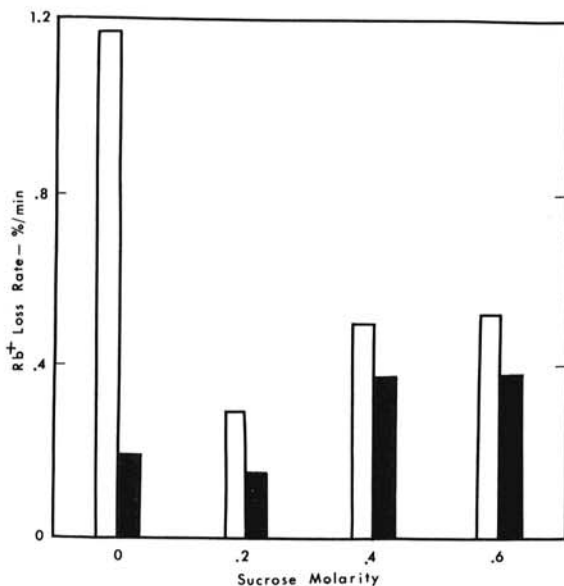


Fig. 5. The effect of plasmolysis on the leakage of $^{86}\text{Rb}^+$ from potato tissue treated with endopectate lyase (EC ef_1) from *Erwinia chrysanthemi*. Potato disks were allowed to accumulate $^{86}\text{RbCl}$ (ca. 2.0×10^4 cpm/g tissue) in solutions containing 0, 0.2, 0.4, and 0.6 M sucrose and were rinsed, with RbCl , at the same sucrose concentrations. Disks (0.3 g/ml) were then treated with EC ef_1 lyase (0.4 units/ml) (open bar) or heat-inactivated lyase (solid bar) in a solution containing 10 mM Tris hydroxymethyl aminomethane - HCl buffer, 1 mM CaCl_2 and antibiotics (50 μg penicillin G and 100 μg streptomycin sulfate per ml solution), with RbCl and sucrose at the same concentrations as in the uptake and rinse solutions. Isotope loss ($^{86}\text{Rb}^+$) was measured at intervals after addition of enzyme, and the rate of isotope loss determined for the first 30 minutes of enzyme treatment.

ability to cause electrolyte loss from tobacco pith tissue. Lyase activity and phytotoxicity were coincident in the fractions which eluted at ca. pH 9.5; peroxidase activity was eluted at ca. pH 5.0, but recovery of peroxidase was less than 1.0%.

Since no phytotoxic compound(s) was (were) separated from the pectic enzyme in enzyme digests of isolated plant cell walls, reaction products from pectic enzyme digests of whole tissue were investigated. Peroxidase was released from potato disks by EC ef_1 lyase (Fig. 6-A). Catalase (5.1 units/g of tissue in 120 min) was also released from the cell walls in these reaction mixtures. The supernatant from these reaction mixtures was fractionated on CM Sephadex (Fig. 6-B). Peroxidase, at a concentration comparable to that in tissue digests, was eluted in the void volume; pectate lyase was eluted in a salt gradient at 80 mM NaCl, and was coincident with the only fractions causing increased electrolyte loss from the potato tissue.

When the supernatant from potato tissue digested with EC ef_1 lyase was filtered on Amicon UM-10 membrane (pore diameter 12-14 Å, both pectate lyase and the phytotoxic compound(s) were retained by the membrane. Supernatants were maintained at 4 C under nitrogen during the 2 hours required for filtration and were assayed immediately after they were filtered.

In another type of experiment, two potato slices (2×3 cm, 0.4-mm thick) were held adjacent to one another on opposite sides of a cellulose acetate dialysis membrane. The slice on one side of the membrane was treated with 38 units of EC ef_1 lyase in 40 ml of 50 mM Tris-HCl buffer (pH 8.5) containing 1 mM $CaCl_2$; tissue on the other side of the membrane was in the buffer without enzyme. After the enzyme solution was stirred for 3 hours at room temperature, cell maceration and cell death indices in the disks were determined. Cell maceration and cell death indices were 4.9 and 5, respectively, for the tissue in enzyme solution and 0 for the tissue in the buffer solution. Neither the lyase nor the phytotoxic compound(s) passed through the dialysis membrane. Thus, there was no evidence that a low molecular weight breakdown product from lyase-treated tissue was phytotoxic.

Beet root tissue was resistant to maceration and cellular injury by pectate lyase. Beet tissue (0.3 g/ml) treated with

EC ef_1 lyase (0.15 units/ml) in standard buffer lost 8% of its electrolytes in 45 minutes, and there was no invisible loss of anthocyanin from the cytoplasm of the beet cells. Tobacco pith disks lost 42% of their electrolytes under identical conditions. Solubilization of unsaturated uronides in these reaction mixtures was 3.3 μ moles/g of tissue with tobacco and 0.22 μ moles/g with beet. Beet and tobacco tissue were combined in common reaction mixtures to see if a soluble product(s) produced by the action of pectic enzyme on tobacco pith tissue would cause injury to beet tissue. Total electrolyte loss in reaction mixtures containing both beet and tobacco tissue was 23% in 45 minutes; this amount of electrolyte loss would be expected from tobacco alone. Also, no anthocyanin leakage was detected in these reaction mixtures.

Hydrogen peroxide has been reported to accumulate in plant tissue treated with pectic enzymes (21, 25). Hydrogen peroxide is reported to cause increased permeability of plant cell membranes (39). Experiments were performed to determine the role of hydrogen peroxide in cell injury by EC ef_1 lyase. Hydrogen peroxide production was measured by the procedure of Mussell (25) in reaction mixtures containing potato disks (1.3 g/ml) treated with EC ef_1 lyase (1.5 units/ml). No hydrogen peroxide was detected in lyase-treated potato disks after 90 minutes of incubation.

Mercaptoethanol (0.25 to 25 mM) and sodium diethyldithiocarbamate (0.25 to 2.5 mM) did not affect the loss of electrolytes from tobacco pith tissue (0.2 g/ml) treated with EC ef_1 lyase (0.15 units/ml) in standard buffer. Electrolyte loss in potato disks treated in reaction buffer with 100 mM H_2O_2 was no greater than electrolyte loss from disks in buffer alone (3.5% loss in 60 minutes).

The addition of catalase, which decomposes hydrogen peroxide, to reaction mixtures did not affect the loss of electrolytes from potato disks treated with pectic lyase (Fig. 6-C). Hydrogen peroxide is apparently not involved in cell injury caused by pectate lyase.

DISCUSSION.—The endopectate lyase from *E. carotovora*, as purified by Garibaldi and Bateman (13), was shown to be heterogeneous upon polyacrylamide gel electrophoresis. The purification scheme reported here

TABLE 2. Enzyme characteristics in relation to maceration (M) and cellular death (CD) in potato tissue treated with pectic enzymes

Enzyme ^a	enzyme characteristics					M ^c	CD ^c
	Substrate cleavage	Enzyme mechanism	pH optimum	pI	Net ionic ^b charge		
EC ef_1	endo	lyase	9.0	9.4	pos.	+	+
EC ef_2	endo	lyase	9.0	7.9	neg.	+	+
VA ₁	endo	hydrolase	5.3	9.3	pos.	+	+
VA ₂	exo	hydrolase	6.0	6.5	pos.	±	—
RS	endo	hydrolase	6.0	5.5	neg.	+	+

^aReaction mixtures contained ca. 1.2 g of potato disks in 4 ml of 50 mM buffer. Enzyme units per reaction mixture and buffer for each enzyme were: EC ef_1 , 1.4 units with pH 8.5 Tris-HCl buffer; EC ef_2 , 0.6 units with pH 8.5 Tris-HCl buffer; VA₁, 0.1 units with pH 5.5 citrate-phosphate buffer; VA₂, 0.7 units with pH 6.0 citrate-phosphate buffer; RS, 0.2 units with pH 6.0 phosphate buffer.

^bNet ionic charge is the theoretical ionic charge (positive or negative) of the enzyme molecule in the reaction mixture and was determined from the isoelectric point of the enzymes.

^cAfter 4 hours enzyme treatment: + = maceration or cellular death of more than 80% of the tissue, ± = maceration of less than 20% of tissue, and — = no cell death.

for EC e_1 lyase yielded a homogeneous enzyme as determined by acrylamide gel electrophoresis. This homogeneous isozyme is identical to the enzyme reported by Garibaldi and Bateman with respect to pH optimum, pl, substrate preference, and ability to cause maceration and cell death of potato tissue. The current study is the first to demonstrate that a homogeneous enzyme preparation can cause cell death in plant tissue.

The characteristics of the cell injury caused by this endopectate lyase were similar to those reported for cells injured by other pectic enzymes (47). Injury to plant cells caused by EC e_1 lyase was characterized by a rapid loss of the ability of the plasmalemma to function as a semipermeable barrier. Cells in tissue treated with endopectate lyase lost the ability to accumulate and retain Neutral Red. Electrolyte loss in lyase-treated tissue occurred at a rate significantly greater than that in tissue treated with heat-inactivated enzyme. This increased loss of electrolytes could be detected in as little as 1 minute after the addition of lyase to reaction mixtures, and all available electrolytes were lost from cells within 1 to 2 hours after exposure to 0.35 units of lyase per ml. Loss in fresh weight of enzyme-treated tissue paralleled the loss of electrolytes. This weight loss was essentially equivalent to water loss, because measurements were made before any maceration or sloughing of cells in the tissue was detected, and because the percent weight loss was too large to be accounted for by any cellular constituent other than water. Since water and electrolytes were lost at a similar rate, it is suggested that a general breakdown of the plasmalemma of enzyme-treated cells occurs.

Although it is evident that damage to the plasmalemma of plant cells occurs when tissue is treated with endopectate lyase, prior studies have not revealed whether damage results from a direct interaction between pectic enzymes and the cell membrane or whether damage is a secondary effect of enzyme action on the tissue. None of the characteristics of the pectic enzymes which were examined in this study revealed a basis for a direct interaction between the plasmalemma and pectic enzymes. Although Ruesink (36, 37) reported that positively charged protein molecules interacted with the plasmalemma of *Avena* protoplasts and caused them to

lyse, a variety of pectic enzymes which exhibited negative or positive ionic charge at the pH of the reaction mixtures caused cell death in enzyme-treated tissue. Further, pectic enzymes with different pH optima, isoelectric points, and mechanisms of substrate cleavage were all able to cause cell death in tissue. The only enzyme characteristic necessary for causing cell injury was the ability to degrade α -1,4 linked galacturonide in a random (endo) manner and, thus, cause tissue maceration.

Since plasmolyzed tissue is protected from injury by pectic enzymes, the stability of isolated protoplasts exposed to pectic enzymes is not surprising. Mount et al. (23) suggested that a possible direct interaction between pectic enzymes and the plasmalemma might be prevented if contraction of the plasmalemma during plasmolysis masked potential sites of membrane-enzyme interaction. The fact that EC e_1 lyase caused no significant death of tobacco protoplasts when they were subjected to osmotic stretching, argues against this hypothesis (Table 3). Potential masked sites on the membrane surface should have been exposed as protoplasts expanded in solutions of low osmotic strength, thus allowing interaction between the enzyme and the plasmalemma if potential sites for interaction were masked by plasmolysis. It is possible that lyase-plasmalemma interaction occurs, but causes no damage unless the membrane is under osmotic stress. Experimentally it would be very difficult to eliminate this argument. In view of the current evidence, we favor the position that no significant direct interactions occur between pectic enzymes and the plasmalemma.

Although the possibility of a pectic enzyme-plasmalemma interaction has not been eliminated, our results do not support such a mechanism of injury of plant cells by pectic enzymes. The action of other molecules which damage plant membranes is not inhibited by plasmolysis of plant cells. Protease and phospholipase from *E. carotovora* lysed plasmolyzed cucumber protoplasts (44); detergents and polycationic proteins lysed *Avena* protoplasts (36, 37); filipin lysed isolated protoplasts of *Neurospora* (17); dinitrophenol lysed *Avena* protoplasts (38); and a host specific toxin from *Helminthosporium victoriae* lysed protoplasts from oat varieties susceptible to the fungus but not protoplasts from varieties resistant to the fungus (38). Thus, it would appear that the prevention of membrane interaction between pectic enzymes and plasmalemma by plasmolysis (if it occurs) would be an unusual phenomenon.

Reaction products from the action of enzymes on cell walls do not appear to be responsible for cell injury. Both pectic lyases and pectic hydrolases caused cell death, but reaction products from the action of the two types of enzymes are different. Hydrolases cleave the α -1,4 bond of galacturonic acid polymers to produce a reducing group and a terminal nonreducing end; lyases produce a reducing group and a β -4-5 unsaturated uronide (7). The only common product is the reducing group. When potato tissue was treated with EC e_1 lyase, reaction products accumulated in the reaction mixtures at less than 1.0 μ mole/ml/hour, and this concentration of reducing sugar is not toxic to plant tissue.

Earlier reports that pectic enzymes solubilize host enzymes which are apparently associated with the plant cell wall were confirmed (21, 31). EC e_1 lyase released

TABLE 3. Death of tobacco protoplasts subjected to osmotic stretching during exposure to endopectate lyase (EC C_1)

Osmoticum molarity	protoplast death (%) ^a			
	10 minutes ^b		90 minutes ^b	
	E	B	E	B
0.63	0	0	4	0
0.51	7	7	14	6
0.40	6	17	10	30
0.29	40	47	54	61

^aProtoplast death (%) determined on counts of ca. 100 protoplasts. Differences between enzyme treatments and buffer treatments are not significant ($P=0.05$) except for the 0.4 M, 90 minute treatment.

^bReaction mixtures contain ca. 6×10^3 protoplasts in 1.0 ml of 50 mM Tris-HCl buffer (pH 8.5) with mannitol osmoticum at the indicated concentrations and 1.8 units of EC C_1 lyase (E) or buffer controls (B).

peroxidase and catalase from both isolated cell wall material and intact tissue, but neither of these enzymes was phytotoxic. EC ef_1 lyase reaction products were fractionated by either isoelectric focusing or column chromatography on CM Sephadex at pH 8.5; when enzyme digests from tobacco wall or potato tissue treated with EC ef_1 lyase were fractionated, phytotoxic activity was detected only in fractions containing the residual endopectate lyase activity. With the procedures employed, a phytotoxic enzyme released by lytic digestion of wall or tissue should have been detected unless the enzyme was labile or had an unusually high isoelectric point similar to that of EC ef_1 lyase (ca. 9.4).

No low molecular weight phytotoxic product was detected in digests of tissues treated with pectic enzymes. When two potato slices were held adjacent to one another on opposite sides of a cellulose acetate dialysis membrane, with one slice in EC ef_1 lyase and the other in buffer, no injury to the disk in buffer was detected. For the tissue in buffer to have been injured, a low molecular weight breakdown product would have to diffuse only through the thickness of the membrane (ca. 0.07 mm).

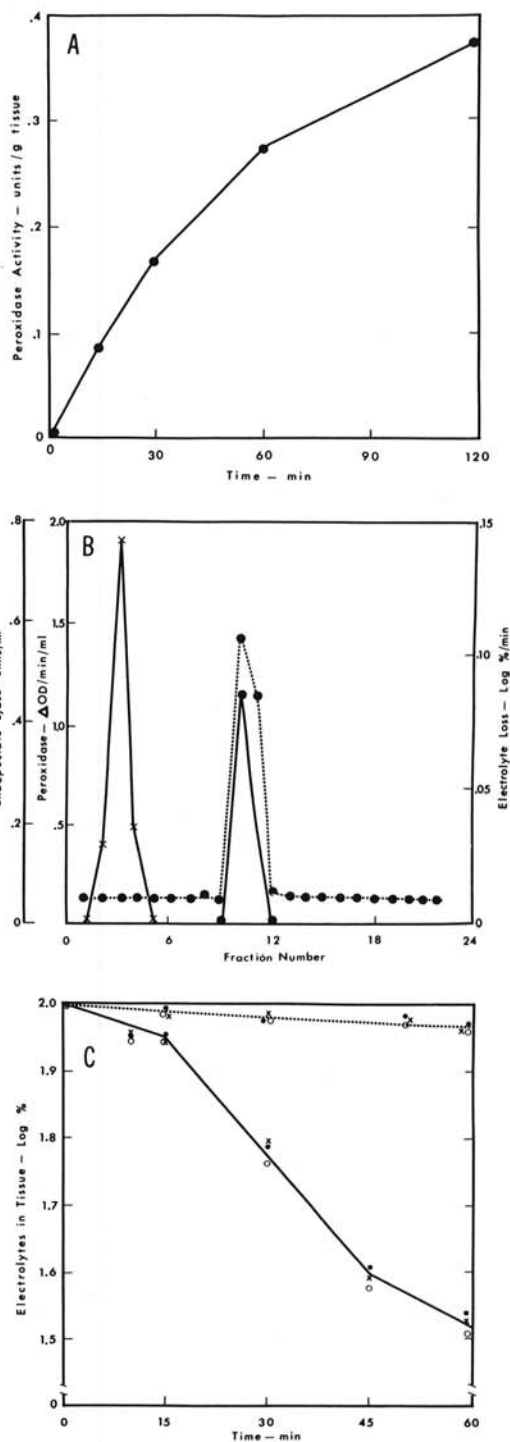
Beet root tissue is resistant to maceration by pectate lyase from *E. aroidea* (46) and to maceration and killing by endopolygalacturonase from *R. stolonifer* (40). If, in fact, the death of plant cells treated with pectic enzymes is due to the production of a soluble breakdown product during wall degradation, then beet cells would be resistant to injury by pectic enzymes because there would be no wall breakdown to generate toxic compounds. If beet tissue were incubated with pectic enzyme in the presence of a tissue injured by the enzyme, the beet cells would be

exposed to any soluble degradation products which were generated by digestion of walls of the susceptible tissue. Beet tissue incubated with EC ef_1 lyase and tobacco pith tissue was not injured even though the tobacco tissue was macerated and killed.

Hydrogen peroxide did not accumulate in potato tissue treated with EC ef_1 lyase, although hydrogen peroxide is

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Fig. 6—(A to C). A) Release of peroxidase from potato tissue treated with endopectate lyase (EC ef_1) from *Erwinia chrysanthemi*. Potato disks (1.0 g/ml) were incubated with EC ef_1 lyase (0.15 units/ml). At intervals after the addition of enzyme, samples of the supernatant were withdrawn and assayed for peroxidase activity by the *o*-dianisidine assay. No peroxidase was released from tissue treated with heat-inactivated lyase. B) Carboxymethyl (CM) Sephadex chromatography of an endopectate lyase (EC ef_1) digest of potato tissue. Potato tissue (0.75 g/ml) was incubated 3 hours at 25 C with EC ef_1 lyase (0.6 units/ml). The digest was centrifuged at 10,000 g for 10 min. The supernatant fluid (5 ml) was applied to the bed of a CM Sephadex column (0.9 × 12.5 cm) equilibrated with 50 mM Tris-HCl buffer, pH 8.5. The sample was eluted at 0.2 ml/minutes with 10 ml of the Tris buffer, followed by a 30 ml linear concentration gradient of NaCl in the Tris buffer (NaCl = 0-0.4 M). Two ml fractions were collected and dialysed. Peroxidase activity (—X—X—) was measured by the guaiacol assay. Residual pectate lyase activity (—●—) was measured by the 230 nm absorbance assay. Cellular injury (----) was measured as electrolyte loss from potato disks treated with 1.0 ml of each fraction. C) Effect of catalase on the loss of electrolytes from potato tissue treated with endopectate lyase (EC ef_1) from *Erwinia chrysanthemi*. Potato tissue (1.3 g/ml) was incubated with EC ef_1 lyase (1.5 units/ml) in 4-ml reaction mixtures with the addition of 0 (o), 1000 (X), or 5000 (●) units of catalase (Worthington Biochemical Corp., Freehold, N.J.). Electrolyte loss was measured as the log of % electrolytes remaining in the tissue (——). Log % electrolytes remaining in tissue treated with heat-inactivated enzyme (----) did not fall below 1.97 (6% electrolyte loss) for any treatment. Catalase did not have a significant effect ($P = 0.05$) on electrolyte loss.



produced in cauliflower and cotton tissue treated with pectic enzymes (21, 25). Catalase at concentrations sufficient to degrade 1.5 μ moles H_2O_2 /ml/minute did not protect potato tissue from injury by the lyase. Hydrogen peroxide at concentrations 1,000-fold greater than those reported to accumulate in cotton tissue treated with endopolygalacturonase (25) did not injure potato tissue. Siegel and Halpern (39) reported that a variety of antioxidants (at 0.1 mM) inhibited damage of beet cell membranes by hydrogen peroxide. Cell injury in tobacco tissue treated with EC ef₁ lyase was not inhibited by an antioxidant, mercaptoethanol (25 mM) or by an inhibitor of free radical reactions, diethyldithiocarbamate (2.5 mM). Thus, hydrogen peroxide does not appear to be associated with injury of potato or tobacco tissues by EC ef₁ lyase. No evidence was obtained to support the hypothesis that cell injury in tissue treated with endopectate lyase results from reaction products or by-products generated during breakdown of the plant cell walls.

There was a close correlation between the rate of breakdown of the cell walls in tissue treated with pectic enzymes and the rate of cell membrane damage (electrolyte loss, cell death) in tissue. In this investigation, all enzymes which caused cell death also caused tissue maceration. The exopolygalacturonase from *V. albo-atrum* caused neither cell death nor tissue maceration. When the concentration of this enzyme was increased, small amounts of both maceration and cell injury were detected. Garibaldi and Bateman (13) reported that an isozyme of endopectate lyase (pI 4.6) from *E. chrysanthemi* caused neither maceration nor cell death. Although some investigators have reported that tissue maceration can be detected up to 50% sooner than cell death in some systems (15, 43), there is no report of a pectic enzyme that causes cell death without causing tissue maceration.

Since there is no evidence that either a pectic enzyme-plasmalemma interaction occurs or that breakdown products from enzymatic degradation of cell walls are involved in cell injury, and since there is an apparent correlation between wall breakdown and cell injury, it seems likely that membrane damage results from enzymatic damage to the cell wall. Damage to the plasmalemma would occur if the enzymatically degraded wall could not support the plasmalemma under the conditions of osmotic stress generated in a hypotonic solution (15, 47). All of the results obtained in this investigation are consistent with the hypothesis that membrane damage in cells of tissues treated with pectic enzymes results from a loss in ability of enzymatically degraded plant cell walls to support the limiting membrane of the cell. Additional studies which support this hypothesis are being published elsewhere (2).

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