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Polygalacturonic Acid *trans*-Eliminase in the Osmotic Shock Fluid of *Erwinia rubrifaciens*: Characterization of the Purified Enzyme and Its Effect on Plant Cells

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An endopolygalacturonic acid *trans*-eliminase (EC 4.2.2.2), released by osmotic shock of *Erwinia rubrifaciens* cells, has been purified to near homogeneity (3,100-fold) by column chromatography on diethylaminoethyl-cellulose, phosphocellulose, and hydroxyapatite-cellulose followed by isoelectric focusing. It has a molecular weight of 41,000, $s_{20,w}$ of 3.09S, an isoelectric point of pH 6.25, pH optimum of 9.5, and a temperature optimum of 37 C and requires Ca^{2+} with an optimum concentration of 0.5 to 1.0 mM. Mg^{2+} could not substitute for Ca^{2+}. Tyrosinyl residues seem essential for enzyme catalysis based on rapid inactivation by tetranitromethane. The enzyme prefers unmethylated polygalacturonic acid as the substrate, cleaving α-1,4-glycosidic linkages randomly to form unsaturated galacturonides at a V_{max} of 1,166 μmol of product/min per mg of protein and a K_m of 5 mg of polygalacturonic acid per ml. Over 90% of the enzyme activity is released from osmotically shocked *E. rubrifaciens* cells. Unlike *E. rubrifaciens*, *trans*-eliminase is not released from *Erwinia carotovora* cells by osmotic shock treatment, but enzyme activity is detected in the culture medium. The release of the enzyme is reduced fivefold by the addition of dibutyryl cyclic adenosine 5'-monophosphate. The hypersensitive reaction in tobacco leaves was induced within 60 min after injection of less than 1 μg of purified *E. rubrifaciens* *trans*-eliminase. Single cells of tobacco in suspension culture are readily killed by the enzyme, whereas tobacco protoplasts remain unaffected when treated in the same manner. These results indicate that endopolygalacturonic acid *trans*-eliminase is a constitutive enzyme possibly located in the periplasmic space of the *E. rubrifaciens* cell and releases enzyme into the culture medium in the presence of substrate. The release of the enzyme in tobacco tissue and the *trans*-eliminative cleavage of plant cell wall components may be steps leading to hypersensitivity of the tobacco tissue.

Plant pathogenic species of the genus *Erwinia* are able to elicit the hypersensitive reaction in higher plants (13, 14, 15). This reaction is characterized by the rapid collapse and desiccation of tissues around the site of inoculation (13). Although a number of studies (11, 15, 17, 18, 33) have been conducted in search of the causal agent elaborated by the bacteria, there has been no satisfactory identification of such compounds.

We wish to report the isolation, purification, and physicochemical and biological characterization of a protein that elicits a hypersensitive-like reaction. The protein causing the hypersensitive reaction occurs in the osmotic shock fluid of *Erwinia rubrifaciens*. The protein causing the hypersensitive reaction has been purified to near homogeneity and has been identified as an endopolygalacturonate *trans*-eliminase with a molecular weight of 41,000. Experiments describing the presence of active components in the osmotic shock fluid of *E. rubrifaciens* appeared in a preliminary report (6).

MATERIALS AND METHODS

Strains. *Erwinia rubrifaciens* strain 6D327, a pathogen of Persian walnut trees, was used in this study. The characteristics of this strain with respect to other enterobacteria are described elsewhere (5). *Erwinia carotovora* strain EC201, a soft rot-causing pathogen of vegetables, was obtained from M. N. Schroth. These strains were maintained on 1.5% agar medium containing 2% glucose, 1% yeast extract, and 2% calcium carbonate (Mallinckrodt ultrafine). Cells of these strains were grown in 4-liter Fernbach flasks containing 1.5 liters of broth medium 523 (12) at 23 C with shaking. The cells were

harvested at late log phase by centrifugation in a Sharples continuous-flow centrifuge.

Osmotic shock procedure and enzyme isolation. Cells of *E. rubrifaciens* were washed twice with 0.03 M Tris-chloride buffer, pH 7.6, and resuspended in 40 volumes of the same buffer. This was followed by rapidly mixing in an equal volume of 40% sucrose (wt/vol) in 0.03 M Tris-chloride–1 mM sodium ethylenediaminetetraacetate buffer, pH 7.6. The mixture was stirred for 10 min, and the cells were collected by centrifugation at 15,000 × *g* for 30 min. The pelleted cells were thoroughly drained and then resuspended in 100 volumes of ice-cold 0.1 mM $MgCl_2$. The suspension was then centrifuged for 2 h at 15,000 × *g*, and the supernatant, containing the osmotic shock fluid, was collected and stored at 4 C for no more than 12 h if not used immediately. Diethylaminoethyl-cellulose (Whatman DE-52) was washed with 0.5 M NaOH followed by distilled water until neutral and then with 1.0 M HCl and distilled water until neutral and finally was equilibrated with 0.03 M Tris-chloride, pH 7.8. The material in the supernatant was adsorbed onto a degassed column (2 by 14 cm) of the cellulose resin at a load proportion of less than 30 mg of protein per g of resin. The column was washed with 250 ml of 0.03 M Tris-chloride buffer and then eluted with a linear gradient of KCl (500-ml total) to 0.4 M. The fractions (8 ml/fraction, usually obtained at 0.1 M to 0.15 M KCl) containing the protein that causes the hypersensitive reaction (i.e., *trans*-eliminase activity) were combined (1.4 to 1.6 mg of protein) and dialyzed against 0.05 M Tris-chloride, pH 7.85. The dialyzed material was adsorbed on a column (1.5 by 8.0 cm) of phosphocellulose (Whatman P-11) preequilibrated with 0.05 M Tris-chloride, pH 7.85. The column was then washed with 200 ml of Tris buffer containing 0.05 M KCl and eluted with a 400-ml linear KCl gradient to a concentration of 0.5 M. The active fractions (6 ml each), which elute at about 0.15 M KCl, were combined (42 ml) and dialyzed overnight against 0.05 M potassium-phosphate buffer, pH 7.0. The material was adsorbed on a column (1.5 by 6 cm) of hydroxyapatite-cellulose (ratio of 10 g of hydroxyapatite [Bio-Rad Laboratories, Richmond, Calif.] to 1 g of Whatman CF-11 cellulose powder) preequilibrated with the phosphate buffer. The column was then washed with 200 ml of phosphate buffer and developed by a 200-ml linear phosphate gradient from 0.05 M to 0.35 M potassium phosphate. The enzyme, eluted at about 0.15 M potassium phosphate, was purified some 2,000-fold over the original crude shock protein preparation. The enzyme was further purified by isoelectric focusing on a 110-ml capacity LKB 1800 focusing column with a 0 to 60% glycerol gradient containing 1% ampholines with a pH range of 5 to 8. In later purification experiments preparations of higher specific activity (450 μmol/min per mg versus 337 μmol/min per mg) were obtained by omitting the hydroxyapatite step and proceeding directly to the isoelectric focusing step. Enzyme was focused for 3 days at a power output of 1 W or less.

Assay of endopolygalacturonate *trans*-eliminase. The periodate-thiobarbiturate (36) and the optimal absorbance (26) methods were employed. The standard reaction mixture consisted of 0.1 M glycine-sodium hydroxide (pH 9.25)–0.5 mM $CaCl_2$ and 0.5% (wt/vol) of substrate in a total volume of 2.0 ml. Unless mentioned otherwise, the substrate was polygalacturonic acid (Sigma Chemical Co., St. Louis, Mo.) set at pH 9.0 with NaOH. The absorbance at 235 nm was continuously monitored in a Beckman ACTA III recording spectrophotometer equipped with thermostatically controlled cuvette holders. Unless stated otherwise, reactions were conducted at 37 C, and the initial reaction rates were measured. One unit of polygalacturonate *trans*-eliminase activity is defined as the formation of 1 μmol of unsaturated galacturonide/min per mg of protein at 37 C, using an extinction coefficient of 4,600 at 235 nm.

Total pectinase activities were measured by the loss in viscosity of 0.5% (wt/vol) sodium polypectate or 0.5% (wt/vol) pectin N. F. (Sunkist Growers, Los Angeles, Calif.) in Ostwald-Fenske viscometers (size 300).

Reducing sugars were measured with Nelson reagent (28). Protein was determined by the method of Lowry et al. (19).

Analytical centrifugation. Rate sedimentation was performed in a Beckman model E analytical ultracentrifuge equipped with photoelectric scanning optics employing a double-sectored capillary-type aluminum-Epon cell with sapphire windows in an An-D type rotor. All preparations of endopolygalacturonate *trans*-eliminase were dialyzed extensively against 0.1 M NaCl in 0.001 M Tris-chloride, pH 8.0, before centrifugation. The molecular weight of the enzyme was determined by sedimentation equilibrium in the same instrument employing the meniscus depletion procedure originally described by Yphantis (37) and modified by Chervenka (2). After equilibrium was reached (15 h), photoelectric scans at 280 nm were taken. The molecular weight was calculated by following optical density by photoelectric scanning as described by Chervenka (2). The partial specific volume of the enzyme was calculated from its amino acid composition (J. M. Gardner and C. I. Kado, unpublished observations).

Gel electrophoresis. Molecular weight of the enzyme was also estimated by electrophoresis in 7.5% acrylamide gels containing sodium dodecyl sulfate (SDS) (35). Phosphorylase A (96,000), bovine serum albumin (68,000), ovalbumin (44,000), and trypsin (24,000) were used as molecular weight standards. Gels were stained with Coomassie blue, destained at 40 C, and stored in 7.5% acetic acid in 5% methanol (vol/vol).

Sedimentation analysis in sucrose. The enzyme preparations were examined by rate sedimentation in 7 to 16% (wt/vol) sucrose density gradients containing 0.1 M potassium phosphate, pH 7.5, as described elsewhere (21). The volume of the gradient was 12 ml, and centrifugation was performed in a Spinco SW41 Ti rotor at 40,000 rpm for 16 to 22 h.

Hypersensitivity and protoplast assays. The hypersensitivity test was performed as described by Klement et al. (14) using young tobacco (*Nicotiana tabacum* cv. Havana) plants possessing 8 to 10

leaves. Only the leaves situated on the middle part of the plant were chosen.

Single-cell cultures of tobacco were obtained from the U.S. Western Regional Laboratory, Richmond, Calif. Protoplasts were prepared essentially by the procedure of Motoyoshi et al. (24).

RESULTS

Purification of endopolygalacturonate *trans*-eliminase (protein causing the hypersensitive reaction). Table 1 summarizes the purification of the enzyme from osmotic shock fluid of *E. rubrifaciens*. A considerable degree of purification is already achieved by employing osmotic shock, which is known to selectively release a number of degradative enzymes from *Escherichia coli* cells (10, 29).

Analysis of the *E. rubrifaciens* shock fluid reveals at least 15 distinct proteins in SDS-acrylamide gels (Fig. 1a). After three chromatographic steps and one isoelectric focusing step of pooled protein samples, a single near-homogeneous protein was obtained (Fig. 1b). A purification of 3,100-fold over the activity in the osmotic shock fluid was achieved. Enzyme of very high specific activity (450 units per μg of protein) was obtained in the final step by isoelectric focusing. The final enzyme preparation represented 400 μg of near-homogeneous protein, as judged by SDS-acrylamide gel electrophoresis (Fig. 1b), rate, and equilibrium centrifugation analyses (see below).

Molecular weight. The molecular weight of the enzyme was estimated by three separate means: velocity sedimentation in sucrose density gradients, equilibrium analytical centrifugation, and SDS-gel electrophoresis. The average molecular weight obtained by velocity sedimentation in sucrose density gradients was 41,500 (Fig. 2). Equilibrium analytical centrifugation analyses gave a slightly lower estimate of near 39,000 when calculated from a partial specific volume ($\bar{v}$) of cm^3/g based on the amino acid composition of the enzyme. The enzyme is homogeneous as judged by the straight-line plot of the log optical density (A_{280}) as a function of radial displacement in the An-D rotor (Fig. 3). The enzyme has an $s_{20,w}$ of 3.09S. An additional molecular weight estimate of 41,000 was obtained from analyses in SDS-acrylamide gels and compares well with the above values (Fig. 4).

pI. The isoelectric point of the enzyme is pH 6.25, as determined by isoelectric focusing (Fig. 5).

pH optimum. *E. rubrifaciens* endopolygalacturonate *trans*-eliminase displays a relatively high pH optimum of pH 9.5. Such high pH optimum is characteristic of pectic *trans*-eliminases of other bacteria (3, 4, 7, 8, 20, 22, 27, 30). The enzyme is relatively inactive at pH 7.0.

Temperature optimum. The enzyme has a temperature optimum of 37 C, as determined from the linear reaction rate kinetics within 2 min after enzyme is added to substrate.

Thermal stability. Enzyme preparations showed no appreciable loss in activity when stored for 8 to 12 weeks at −20 or −70 C. Heating the enzyme at 80 C for 30 s destroyed 80% of the activity, and 90% loss of activity occurred after 60 s. Glycerol (20%) had no effect on stabilizing dilute solutions of the enzyme.

Substrate preference. *E. rubrifaciens* endopolygalacturonate *trans*-eliminase prefers unmethylated polygalacturonic acid as the substrate (Fig. 6). Intermediate activity is obtained with sodium polypectate, and highly es-

TABLE 1. *Purification of E. rubrifaciens endopolygalacturonate trans-eliminase*

Step[a]	Vol (ml)	Total protein (mg)[b]	No. of units of activity[c]	Sp act (units/mg)	Fold purified
1. Shock protein	1,720.0	1,462	212	0.145	–
2. Diethylaminoethyl-cellulose	73.5	22.4	196	8.75	60
3. Phosphocellulose	70.3	1.4	126	90.0	621
4. Hydroxyapatite-cellulose	40.3	0.177	59.7	337.3	2,326
5. Isoelectric focusing	12.0[d]	0.400[d]	180[d]	450.0	3,100

[a] Purification from 49 g of cells (fresh-packed weight).

[b] Protein was determined by the method of Warburg and Christian (34) for steps 1, 2, and 3 and by the method of Lowry et al. (19) for steps 4 and 5.

[c] A unit of activity is defined as the formation of 1 μmol of unsaturated galacturonide/min per mg of protein at 37 C using an extinction coefficient of 4,600 at 235 nm.

[d] From enzyme obtained by combining several chromatographic preparations of a previous step (either step 3 or 4).

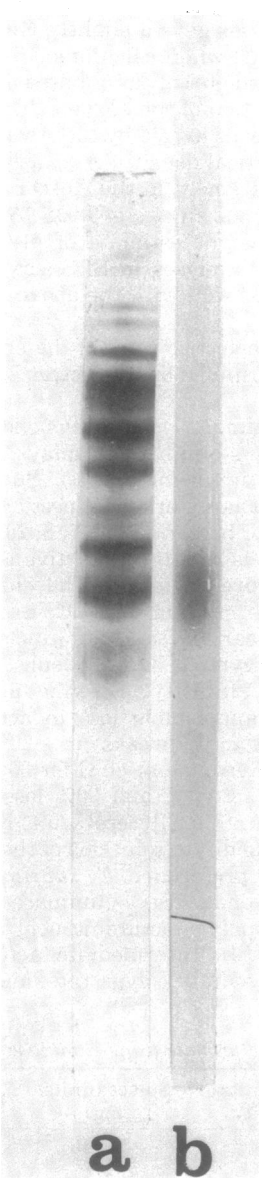

FIG. 1. *(a) Separation of proteins in the osmotic shock fluid of E. rubrifaciens by electrophoresis in 7.5% acrylamide gel containing SDS (35). (b) SDS-acrylamide gel electrophoresis of purified endopolygalacturonic acid trans-eliminase isolated from the osmotic shock fluid of E. rubrifaciens. The piece of copper wire marks the point of migration of the tracking dye. The enzyme was stained with Coomassie blue.*

terified pectin is the least preferred of the three substrates.

K_m and V_{max}. The Michaelis constant (K_m) of the enzyme is approximately 5 mg of polygalacturonic acid per ml (Fig. 7). The maximum potential difference (V_{max}) is 1,166 μmol of unsaturated galacturonide/min per mg of protein.

Nature of polymer cleavage. The enzyme randomly cleaves α-1,4-glycosidic bonds by *trans*-elimination as judged by the rapid decrease in viscosity relative to the production of unsaturated galacturonide [0-(4-deoxy-β-L-5 *threo*-hexopyranos-4-enyluronic acid)-(1 $\rightarrow$ 4)-D-galacturonic acid] (Fig. 8).

Requirement for Ca^{2+} and effects of other cations. The enzyme has an absolute Ca^{2+} requirement and a concentration optimum of 0.5 to 1.0 mM (Fig. 9). The enzyme is inactive without Ca^{2+}, and as expected ethylenediaminetetraacetate also inactivated the enzyme. Mg^{2+} could not be substituted for Ca^{2+}, and Mg^{2+}, Na^+, and K^+ were inhibitory at a concentration of 5 mM in reaction mixtures containing optimal amounts of Ca^{2+}.

The tyrosyl residue is essential for *trans*-eliminase activity. Various reagents that probe polypeptides were tested against the activity of the enzyme (Fig. 10). Dinitrofluorobenzene inhibited endopolygalacturonate *trans*-eliminase activity when 0.5 mM reagent was reacted with enzyme for 5 min. To test whether or not sulfhydryl groups were necessary for the activity of the enzyme, 10 mM *N*-ethylmaleimide was used. Endopolygalacturonate *trans*-eliminase activity was not inhibited by *N*-ethylmaleimide. To test if tyrosine was specifically involved, tetranitromethane, a reagent for tyrosine in proteins (31), inhibited the enzyme at very low concentrations (Fig. 11). The reaction appears to be nitration of the tyrosyl moiety, since at low pH, when oxidation of cysteine is likely to occur, no inactivation was observed (Fig. 11). Sensitivity to iodine-potassium iodide supported this hypothesis (Fig. 10), and therefore tyrosyl moieties of the enzyme seem to provide a crucial functional role. Trinitrobenzenesulfonic acid (10 mM), a reagent which attaches to amino groups, only reduced activity by 5 to 10% when the reagent was reacted with enzyme for 5 min. We estimate one to two tyrosine residues per molecule of endopolygalacturonate *trans*-eliminase (Gardner and Kado, unpublished observations).

Release of endopolygalacturonate *trans*-eliminase. Figure 12 shows that over 90% of the enzyme activity occurs in the osmotic shock fluid of *E. rubrifaciens* cells that were grown on

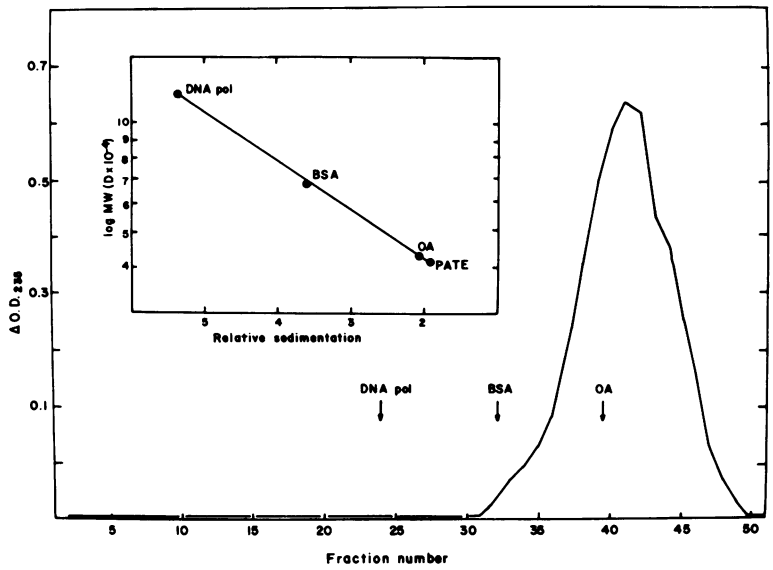

FIG. 2. *Velocity sedimentation analysis of endopolygalacturonic acid trans-eliminase (PATE) in sucrose density gradient. Resolution of 1.5 μg of enzyme in a 7 to 16% (wt/vol) sucrose linear gradient containing 10% ethylene glycol (vol/vol) and 0.1 M potassium phosphate buffer, pH 7.5, by centrifugation (40,000 rpm) for 21 h at 4 C in a Spinco SW41 rotor. Fractions (240 μl) were taken from the bottom of the tube, and each fraction was assayed for enzyme activity and for protein markers: deoxyribonucleic acid polymerase α (Vinca rosea), bovine serum albumin (BSA), and ovalbumin (OA).*

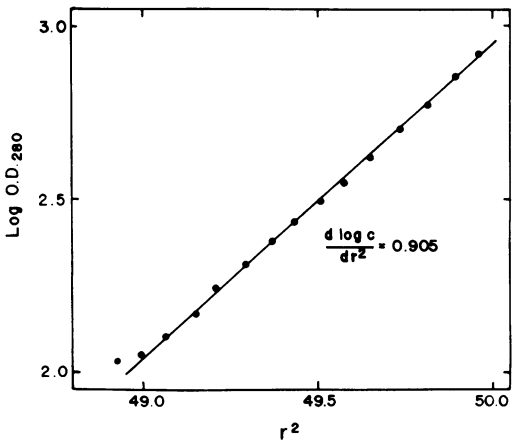

FIG. 3. *Endopolygalacturonic acid trans-eliminase (specific activity, 450 μmol/min per mg) at sedimentation equilibrium, 30,000 rpm, 5 C, using 0.1 M NaCl-0.001 M Tris-chloride, pH 8. The $\log_{10}$ of the optical density is plotted against the square of the radial distance in the rotor.*

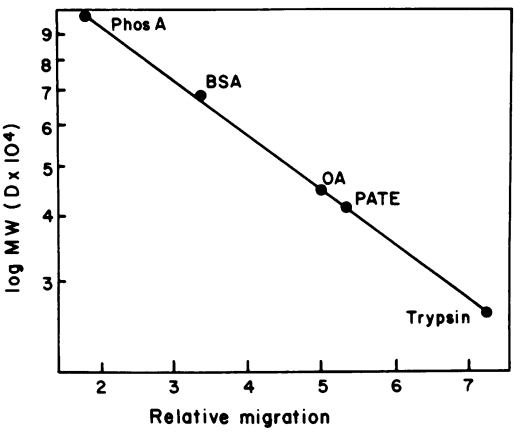

FIG. 4. *Molecular weight estimates by SDS-acrylamide gel electrophoresis of endopolygalacturonic acid trans-eliminase (PATE) relative to marker proteins: phosphorylase A (Phos A), bovine serum albumin (BSA), ovalbumin (OA), and trypsin.*

sucrose. Less than 20% of the activity occurs in the osmotic shock fluid when cells are grown on pectin or polygalacturonic acid, and less than 10% of the enzyme activity was released by sonication of osmotically shocked cells grown on sucrose. When cells were grown on polygalacturonic acid, the culture medium contained about 85% of the total enzyme activity; the remaining 14 to 15% of the enzyme activity was

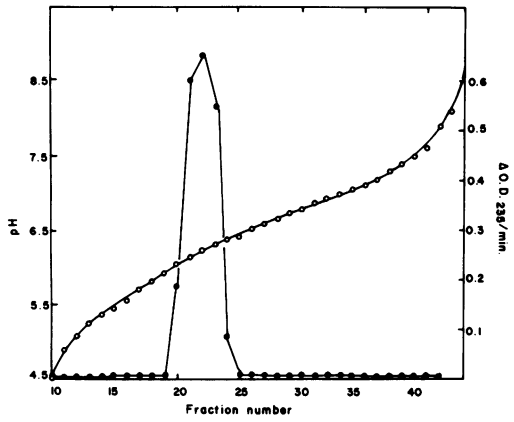

FIG. 5. *Isoelectric focusing of endopolygalacturonic acid trans-eliminase. Enzyme, placed in the central region of a 0 to 60% glycerol gradient containing 1% ampholines (pH range, 5 to 8), was focused for 3 days, and 1.8-ml fractions were collected and assayed for pH (open circles) and enzyme activity (filled circles).*

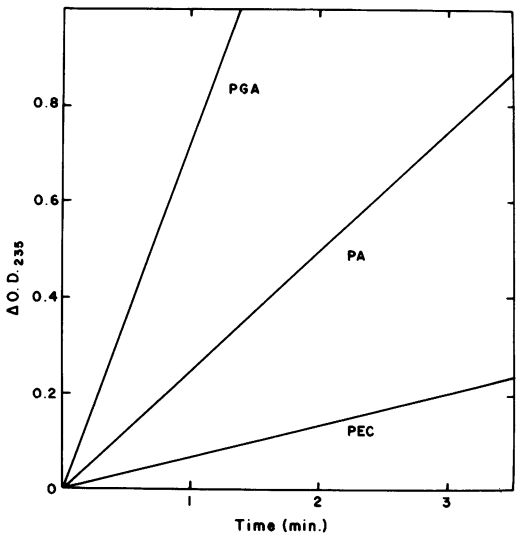

FIG. 6. *Degradation rates of pectic substrates with different degrees of methylation. Abbreviations: PGA, Unmethylated polygalacturonic acid; PA, pectic acid; and PEC, pectin. Reactions were carried out at 0.5% substrate concentration, pH 9.2.*

found in the extract from the cells that had been shocked and then broken by sonication. In the presence of pectin, about 50% of the enzyme activity was released into the medium, and the remaining enzyme occurred intracellularly, except for a very small amount of activity released by osmotic shocking before sonic treatment.

These results suggest that *E. rubrifaciens* is constitutive for the enzyme. In the absence of pectic substrates, it is assumed here that the enzyme is located in the periplasmic space between the cell wall and membrane based on the above osmotic shock experiments.

As a comparative control, *E. carotovora*, a well-known producer of pectic enzymes, was similarly examined for pectinolytic *trans*-eliminases in the osmotic shock fluid. In contrast to *E. rubrifaciens* the enzyme was not released by osmotic shock treatment; however, the enzyme was released into the culture medium. *E. carotovora* cells grown in medium containing sodium polygalacturonate produced about 3- to 10-fold more enzyme than cells grown without this

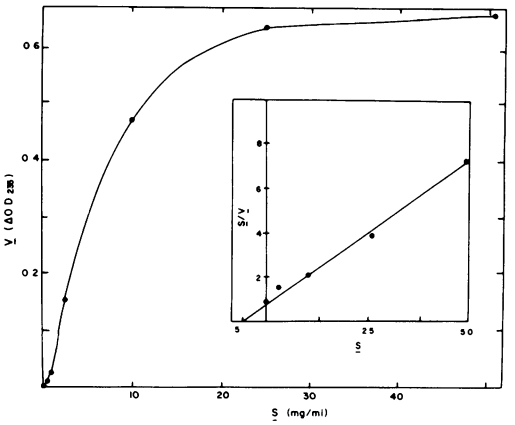

FIG. 7. *Plot of initial velocity, v, versus polygalacturonic acid substrate concentration, S. Woolf plot (insert) shows the relationship between v and S.*

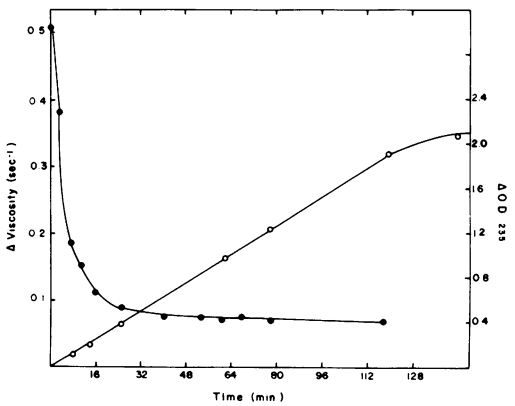

FIG. 8. *Relationship between viscosity change and formation of unsaturated galacturonide. Standard reaction conditions were used with 0.5% sodium polypectate as substrate. Viscosity (filled circles) and absorbancy (open circles) at 235 nm were monitored in identical reaction mixtures.*

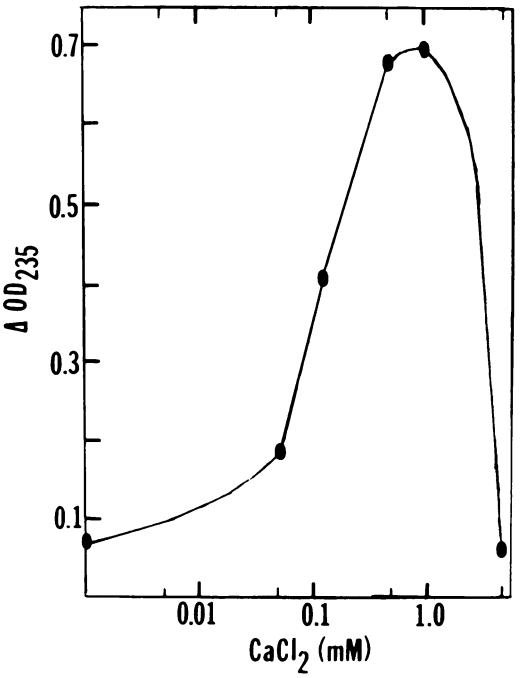

FIG. 9. *Calcium ion requirement and optimum concentration for endopolygalacturonic acid trans-eliminase. Calcium chloride concentration is plotted logarithmically against enzyme activity.*

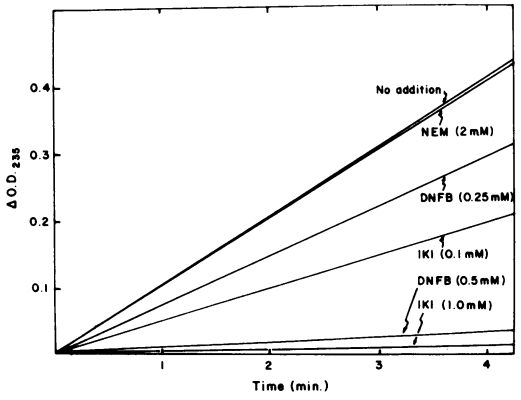

FIG. 10. *Effect of various chemical probes on endopolygalacturonic acid trans-eliminase activity, using N-ethylmaleimide (NEM), dinitrofluorobenzene (DNFB), and iodine-potassium iodide (IKI). Ten microliters of inhibitor (2× concentration) was mixed with 10 μl of enzyme, incubated for 5 min at 23 C, and assayed for trans-eliminase activity.*

substrate. When we grew *E. carotovora* cells in the presence of polygalacturonate and dibutyryl cyclic adenosine 5'-monophosphate instead of polygalacturonate alone, the release of the enzyme was reduced fivefold, and the intracellular concentration of enzyme was increased twofold (Fig. 13). These comparative studies show that the enzyme is constitutive in both species of *Erwinia* and that the retention of the enzyme differs between *E. carotovora* and *E. rubrifaciens*. The effect of dibutyryl

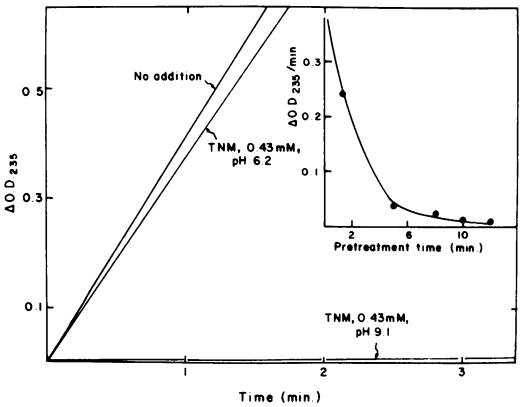

FIG. 11. *Effect of tetranitromethane (TNM) on endopolygalacturonic acid trans-eliminase activity. Ten microliters of 0.86 mM TNM was mixed with 10 μl of enzyme for 5 min at 23 C and then assayed for trans-eliminase activity.*

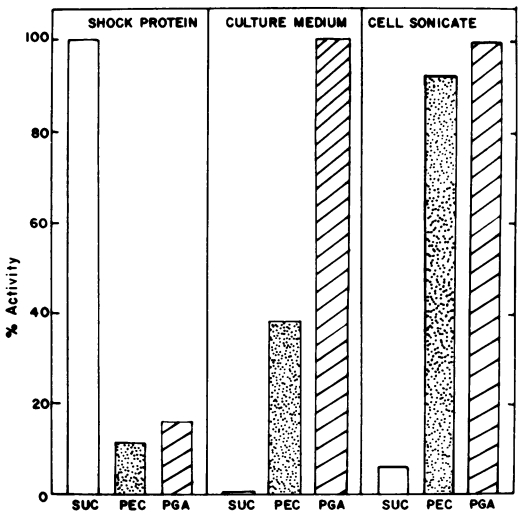

FIG. 12. *Relative levels of endopolygalacturonic acid trans-eliminase activity in osmotic shock fluid (shock protein), in the culture medium, and in the cells after growth of Erwinia rubrifaciens, using sucrose (SUC), pectin (PEC), or polygalacturonic acid (PGA) as the substrate. The percentage of activity represents trans-eliminase activity relative to the highest activity observed in each fraction prepared from cells grown on the specified substrate. The enzyme assay was performed on equivalent amounts of cells (wet weight) and supernatant.*

cyclic adenosine 5'-monophosphate indicates that *trans*-eliminase production may be catabolically repressed.

Maceration and lysis of plant cells by endopolygalacturonate *trans*-eliminase. The hypersensitive reaction, which is elicited in plant tissue by infiltrating the tissue with bacterial suspensions of various incompatible phytopathogenic bacteria (13, 14, 15), can also be induced by infiltrating osmotic shock fluid of *E. rubrifaciens*. Upon analysis of the components in the shock fluid, the active component was found to be endopolygalacturonate *trans*-eliminase. As with bacterial-induced hypersensitivity, where there is loss of host cell selective permeability (15, 25), the enzyme also causes such losses in selective permeability. Increased electrolyte efflux was detected within 60 min after infiltration of the enzyme (500 μg of shock protein or 1 μg of purified enzyme) into the leaf panels of tobacco *(Nicotiana tabacum)* (Fig. 14).

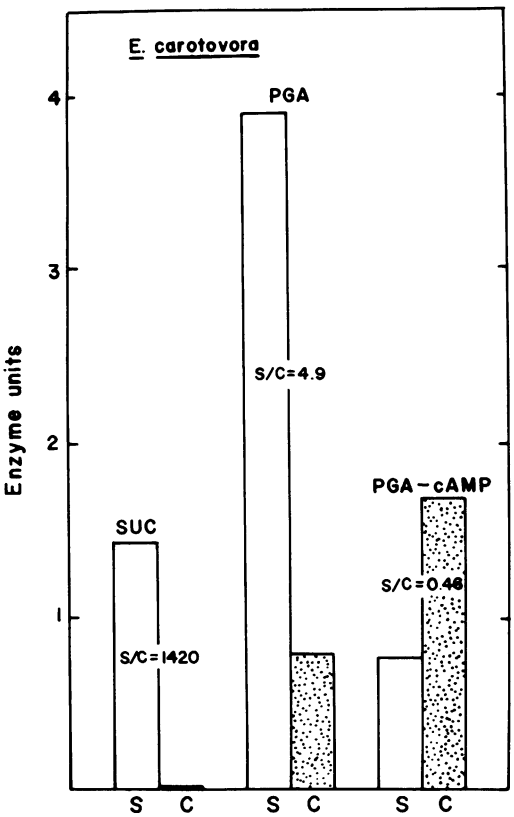

FIG. 13. *Relative levels of endopolygalacturonic acid trans-eliminase of E. carotovora. Extracts obtained by sonication of osmotically shocked cells (S) and culture media (C) were assayed for trans-eliminase activity. This enzyme was not detected in the osmotic shock fluid. The relative amounts of enzyme that occurred intracellularly and in the culture media are expressed as ratios S/C.*

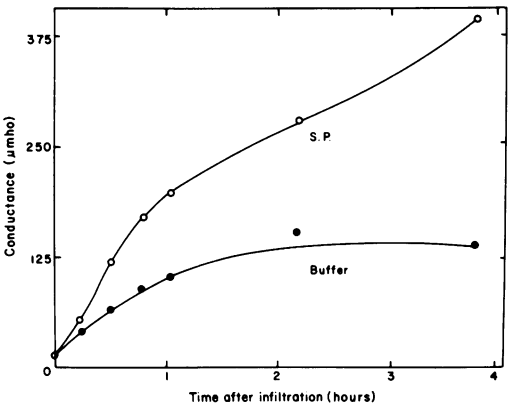

FIG. 14. *Electrolyte loss from tobacco leaf cells exposed to endopolygalacturonic acid trans-eliminase. The loss of electrolytes was measured as follows. Disks (1 cm) were cut from tobacco leaf panels, briefly vacuum infiltrated, and floated on 500 μg of osmotic shock protein per ml (S.P.) for 30 min. The disks were rinsed in distilled water for 15 min, and the conductivity of electrolyte released in the water was measured with a conductance bridge (Radiometer).*

Pectin *trans*-eliminases are known to kill cells during maceration (8, 25). This killing is apparently not due to the immediate direct effect of the macerating enzymes, since they apparently have no visible effects on plant cell protoplasts (32, 33). We also find that purified endopolygalacturonate *trans*-eliminase (>40 μg/ml) has no killing effect on protoplasts of *N. tabacum*. On the other hand, nanogram amounts of the purified enzyme (100 to 1,000 ng) can produce the hypersensitive reaction with subsequent necrosis when injected into the panels of tobacco leaves. Also, single-celled suspensions of tobacco are killed by less than 10 μg of the purified enzyme per ml. These results make it reasonable to attribute cell death to osmotic fragility of the plant cell when exposed to the enzyme, possibly as a result of cell wall loosening or separation.

DISCUSSION

Endopolygalacturonate *trans*-eliminase has been isolated from the osmotic shock fluid of *E. rubrifaciens* and has been purified to near homogeneity. The specific activity of the enzyme is higher than that attained with similar pectinolytic enzymes (3, 4, 8, 9, 22, 23, 27, 30). The pH optimum (pH 9.5), Ca^{2+} requirement, and substrate preference for polygalacturonic acid are comparable to those of partially purified *trans*-eliminases of certain other bacteria (3, 4,

7, 8, 20, 22, 27, 30) and differ from those of oligogalacturonate *trans*-eliminases, which have a neutral pH optimum (9, 23). The isoelectric point is lower than that of endopolygalacturonate *trans*-eliminase of *E. chrysanthemi* (8).

Amino acid analysis of the enzyme indicated an average of one tyrosine residue per molecule of enzyme (Gardner and Kado, unpublished observations). The tyrosine residue appears to be essential for the activity of the enzyme. Although no model has been proposed for its molecular function, one possibility is that the phenolic group of the tyrosyl moiety, which is ionized partially at pH 9.25 (pK of phenolic hydroxyl = 10.01), forms a complex with Ca^{2+}, which in turn engenders conformational changes that are prerequisite for efficient catalysis of substrate. It has been shown that a functional tyrosyl residue, unreactive in native aspartate aminotransferase, becomes highly susceptible to chemical modification during catalysis (1).

We have shown that less than 1 μg of the purified enzyme will kill tobacco leaf cells and produce the hypersensitive reaction. Although polygalacturonic acid *trans*-eliminases are known to macerate plant tissues and cause cell death (8, 25, 30, 33), a relationship between pectic enzymes and hypersensitivity has not been suggested previously. We have presented several correlations between endopolygalacturonate *trans*-eliminase activity and hypersensitivity and have shown that rapid killing of tobacco cells occurs after exposure to the purified enzyme. Tobacco protoplasts remain unaffected by enzyme treatment, but whole single cells of tobacco are killed. Similar observations were reported when cucumber protoplasts were treated with *trans*-eliminase of *E. carotovora* (32). We can tentatively attribute this phenomenon to a disruption of the membrane by osmotic imbalances in plant tissues during cell wall hydrolysis of tobacco cells.

Our results are consistent with the hypothesis that the enzyme is located in the periplasmic space of the *E. rubrifaciens* cell as are a number of degradative enzymes in *E. coli* (10, 29). The enzyme occurs mostly (90%) as an osmotic shock protein when cells are grown in sucrose medium in the absence of polygalacturonic acid. As expected, enzyme levels increase and the enzyme appears in the culture medium in the presence of substrate. The mechanism for the elaboration of the enzyme by the infecting bacteria in plant tissue is unknown. Enzyme located in the periplasmic space could be released following infiltration of the bacterial cells into plant tissue. Once the enzyme is released it comes into contact with substrates in the plant tissue, and the availability of free substrates could promote production and release of more enzyme. The elucidation of these possible mechanisms awaits further study.

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