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## Isolation and partial characterization of the intercellular pectic enzyme complex from *Erwinia carotovora* isolate 14.

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ISOLATION AND PARTIAL CHARACTERIZATION OF THE  
INTRACELLULAR PECTIC ENZYME COMPLEX  
FROM ERWINIA CAROTOVORA ISOLATE 14

A Thesis Presented

by

James Peter Stack

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of the requirements for the degree of

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Department of Plant Pathology


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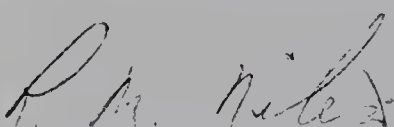
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September, 1978

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## ABSTRACT

The intracellular pectic enzyme complex of Erwinia carotovora isolate 14 comprises 4 pectin depolymerases; PDI, PDII, PDIII and PDIV. PDI has an isoelectric point of pH 9.4, a pH optimum of pH 8.5, and requires  $\text{Ca}^{++}$  for activity. Isoelectric focusing, disc gel electrophoresis, and immunological comparison showed PDI to be the intracellular form of the single extracellular pectin depolymerase PDIIa. PDI and PDIIa showed the unique ability for dual function in depolymerizing sodium polypectate in an endolytic fashion at pH 8.5 in the presence of  $\text{Ca}^{++}$  and in an endohydrolytic fashion at pH 6.0 in the presence or absence of divalent cations. PDII (pI 8.0) and PDIII (pI 6.3) exhibited exolyase activity over a broad range of pH (5.5-9.5) with an optimum at pH 8.5. PDII required  $\text{Mn}^{++}$  for optimal activity and PDIII was enhanced by, but did not require divalent cations. PDIV (pI 6.5) exhibited some exolyase activity as well as some exohydrolase activity, however, its primary function was to convert unsaturated digalacturonic acid into two molecules of unsaturated monomer. A model is proposed to describe the significance of these findings.

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## INTRODUCTION

Cell wall degrading enzymes have been implicated in host-parasite interactions ranging from soft rots to vascular wilts and from tissue deterioration in the field to post harvest decay in storage and transit (7,8). There are several classes of polysaccharide degrading enzymes, each having its own niche in the process of cell wall disintegration (2,13,25). Several researchers have begun to establish the importance of each class of polysaccharidases in pathogenesis. In terms of damage to plant tissue, the enzymes which digest the pectic acid portion of the plant cell wall seem to comprise by far the most important class of polysaccharidases (6,8,9,12,13,56,57,65).

The ultimate goal of most pathological research is to reduce or eliminate the incidence of disease. In order for this goal to be realised, each specific host-parasite interaction must be studied from every perspective. Erwinia carotovora is a phytopathogenic bacterium causing soft rots of many vegetables. Loss of tissue integrity resulting in massive cell lysis and eventual organ destruction are typical of soft rot diseases (12,60). It has been explicitly demonstrated in the host-parasite system, bacterium-potato tuber, that the pathogen secreted pectic enzyme, endo-polygalacturonate trans-eliminase (PGTE) is integrally related to symptom and disease development (39). Since soft rot development is dependent upon pathogen production of PGTE, research concerning the regulation of PGTE synthesis and the characterization of these

enzymes is essential to understanding this disease.

Erwinia carotovora (Jones) Holland isolate 14 has been shown to produce one extracellular pectic enzyme; endo-polygalacturonate trans-eliminase (39,61). When isolated and purified, this enzyme has been shown to induce the necessary changes in potato tuber tissue (electrolyte loss, cellular death and tissue maceration) which result in the soft rot symptoms. Subsequent work has led to the characterization of the regulatory mechanism(s) of this enzyme (22). Interpretation of this work was, in part, based upon the assumption that there was just one intracellular pectic enzyme which corresponded to the one extracellular pectic enzyme.

The purpose of this investigation was to isolate and partially characterize the intracellular enzyme (s) of Erwinia carotovora (Jones) Holland isolate 14 capable of depolymerizing sodium polypectate. From the data obtained in this investigation a model system is hypothesized to explain the importance of the E. carotovora-pectic enzyme complex in the development of soft rot diseases.

## LITERATURE REVIEW

Many researchers have contributed to the extensive yet incomplete body of knowledge concerning the structure and function of plant cell walls. At present it is generally accepted that higher plant cell walls are constructed mainly with polysaccharides and hydroxyproline rich glycoproteins (7,26,29,48). The cell wall is thought to comprise three sectors; the primary wall, the secondary wall, and the middle lamellae. The middle lamellae and the primary wall are formed late in the mitotic cycle, whereas the secondary wall is laid down after cell elongation is complete. Functionally the primary cell wall which is made up primarily of cellulose and hemicellulose, supports and protects the protoplast. The secondary wall provides more rigid support. The middle lamellae, whose primary makeup is pectic acids, serves to hold the primary walls of adjacent cells together.

Pectic acids are polymers of D-galacturopyranose with  $\alpha$ -1,4 linkages (4,59). Also integrated into the long chain there is 1,2-rhamnose which is thought to have polymers of the neutral sugars arabinose and galactose linked to it. Pectic acids are usually classed by the degree of methylation of the polyuronic acid carboxyl groups. Greater than 75% methylation constitutes pectin and less than 75% methylation constitutes pectinic acid (17). The uronic acid residues may also be acetylated at carbons 2 or 3.

Collectively, phytopathogens produce several enzymes capable of attacking the middle lamellae or pectic acid fraction of plant cell

walls (8,55). These enzymes vary in their mechanism of action, hydrolytic or lytic, as well as their mode of depolymerization, endo or exo. The endo enzymes attack the polymer randomly which results in several size (dimer to suspected pentamer) products. The exo enzymes attack the polymer terminally or make some random cuts resulting in varying chain lengths which are then attacked terminally releasing small units from the end (33). The exo hydrolases release galacturonic acid from the polymer, while the exo lyases release unsaturated digalacturonic acid from the polymer (21,49). Another type of pectic enzyme acts to alter the pectic polymer rather than cleave the molecule. Known as pectin methyl esterases, these enzymes demethylate the carboxyl group of the galacturonic acid units (36).

Many fungal polygalacturonases, pectic acid hydrolases, have been extensively characterized (7,8,9,11,42). Usually these fungal enzymes show activity only under acid conditions (pH 4.0-6.5) and do not require metal ions to exhibit activity. Some polygalacturonases have even been reported to be inhibited by calcium (10). The polygalacturonases are involved in many types of host-parasite interactions which differ in disease development and symptom expression. These hydrolases may be involved solely to aid in penetration of the host by the pathogen (1), or they may be involved with other factors which collectively incite disease in the host (the wilt syndrome) (43). In diseases such as soft rots of vegetables, these enzymes are the cause of the most serious effects on the host, namely tissue maceration. When these pectic acid hydrolases are isolated from the pathogen and either purified or used directly, they are still capable

of inducing symptoms in host tissue (41,60).

The trans-eliminases, or pectic acid lyases, are produced by many phytopathogenic bacteria and fungi (8). Cleavage by these enzymes results in the formation of a double bond between carbons 4 and 5 of the uronic acid residue. The formation of this double bond is the basis of the routine assays for this enzyme (37,58,64). Except for a few which attack pectin, the trans-eliminases exhibit greatest activity in the alkaline range (pH 7.5-9.5) (7). In the presence of chelating agents such as ethylenediamine tetraacetic acid (EDTA), the trans-eliminases are inactivated (7,55). The lyase activity is usually greatly enhanced by specific concentrations of calcium. Higher or lower than optimal concentrations of calcium can lead to inhibition of activity (5,34,37). Other divalent cations have been reported to have no inhibitory or stimulatory effect on a trans-eliminase from Erwinia carotovora (37). However, a trans-eliminase from Clostridium multifementans was greatly enhanced by divalent cations other than calcium, with calcium, strontium, and manganese showing the best stimulation, respectively (34). The role of divalent cations in the stimulation of enzyme activity is not clearly understood. It has been suggested that these ions, or at least calcium, predispose the substrate by a conformational change to attack by the enzyme, as well as act as an activator of the enzyme (5,31).

Phytopathogenic species of the genus Erwinia collectively produce a variety of pectic enzymes including endo and exo forms of a trans-eliminase as well as a hydrolase (37,39,46,49). Only trans-eliminases

have been found in Erwinia chrysanthemi (19,54). In certain strains of this organism several isozymes of the lyase separable only by isoelectric focusing have been reported. There was observed to be three main groups of isozymes based upon their isoelectric points (acid, neutral, and alkaline). This grouping was directly correlated with their immunological relationships. Isozymes in the alkaline group were antigenically related to each other but antigenically unrelated to the isozymes in the neutral and acid groups. In each group the isozymes were antigenically related to each other but antigenically unrelated to the isozymes of the other groups (54).

Isolate EC<sub>23</sub> of Erwinia chrysanthemi was found to produce four extracellular polygalacturonic acid trans-eliminases (19), separable only by isoelectric focusing. Isolate 307 of this species produced two extracellular trans-eliminases separable only by isoelectric focusing (18,19). All six of these enzymes showed a pH optima greater than pH 8.0 and demonstrated virtually no activity at pH 6.0 or below. This was determined using assays which measure the formation of double bond or the release of free reducing groups from the substrate. From both strains the enzymes with isoelectric points around pH 9.2 exhibited an endo mode of depolymerization, macerated tissue rapidly, and caused cellular death. From both strains, the enzymes with isoelectric points around pH 8.0 exhibited an exo mode of depolymerization, macerated tissue slowly, and caused cellular death. The first effect that all these enzymes had upon their hosts was electrolyte leakage (18,19).

A Mexican bell pepper isolate of the soft rot bacterium Erwinia carotovora was shown long ago to produce pectic enzymes (28). In culture it produced extracellular pectin methylesterase and extracellular polygalacturonase. The hydrolase, or polygalacturonase, exhibited activity from pH 3.9 to pH 7.9, with optimum activity around pH 5.8 as demonstrated by the release of free reducing groups from the substrate. Subsequent work revealed the mechanisms by which Erwinia carotovora could utilize the breakdown products of the pectic polymers as a food source (27). Galacturonic acid, which is the ultimate breakdown product of polygalacturonase, is converted by isomerization and reduction into a molecule which eventually gets split into pyruvic acid and glyceraldehyde-3-phosphate. Pyruvic acid can then be funnelled into the tricarboxylic acid cycle. Following the discovery of a new enzymatic mechanism for cleaving pectic polymers (3) which showed optimal activity in the alkaline range and resulted in the formation of a double bond between carbons 4 and 5 of the uronic acid residues, Erwinia carotovora isolate 153 was re-examined. It was found and reported to produce a pectin trans-eliminase (58). After full evaluation, it was reported that isolate 153 produced a pectin methylesterase, a hydrolase, and a trans-eliminase (46). These researchers, however, reported great difficulty in separating the hydrolase from the trans-eliminase activity. After several procedures, they were able to obtain a preparation with predominantly hydrolase activity but still demonstrated some trans-eliminase activity.

Further characterization of the trans-eliminase of isolate 153 led to conclusion that there was one extracellular lyase and one intra-

cellular lyase, both having identical properties (37). This was based upon carboxymethylcellulose and DEAE cellulose column chromatographic purified enzymes. There was also reported to be no significant difference in properties between the crude and purified enzymes, excluding stability. These trans-eliminases had a pH optimum of pH 8.5 and required calcium for activity. No other divalent cations showed a stimulatory effect. Initially the lyases released from the substrate products of varying chain length (dimer through pentamer and higher) indicating an endo mode of depolymerization. These products were unsaturated. Ultimately the lyases yielded predominantly unsaturated digalacturonic acid with small amounts of unsaturated trigalacturonic acid, saturated digalacturonic acid, and galacturonic acid. They reported no exo depolymerase activity in this isolate.

Upon application of the intracellular enzyme preparation to the DEAE cellulose column the lyase eluted with the void volume. However, a second pectic enzyme called oligogalacturonic acid trans-eliminase (OGTE) bound to the column and eluted with 0.2 N NaCl (38). The preferred substrate of this enzyme was shown to be unsaturated digalacturonic acid. OGTE exhibited a pH optimum of pH 7.2 and did not require calcium when converting unsaturated digalacturonic acid into two molecules of 4-deoxy-5-hexoseulose uronic acid (unsaturated monomer). OGTE reacted with pectic acid 1/400 the rate of reaction with the unsaturated dimer. It was also shown that the unsaturated monomer could be converted into 2-keto-3-deoxy-D-gluconic acid by cell free extracts, and further metabolized into the food chain.

Erwinia carotovora (Jones) Holland isolate 14 is another soft rot bacterium. After considerable purification (through isoelectric focusing) this isolate was shown to produce one extracellular polygalacturonic acid trans-eliminase with an isoelectric point of pH 9.4 (39,61). Based upon the work with isolate 153, isolate 14 was assumed to have one intracellular lyase identical to the extracellular lyase. This lyase showed a pH optimum of pH 8.5 and required calcium for activity. In highly purified form, it was shown to induce all the symptoms typical of soft rot disease. It induced leakage of electrolytes, followed by tissue disintegration or maceration, and cell death. The death of cells was shown to be a result of the inability of the degraded cell wall to support the protoplast rather than any direct interaction between the pectic enzyme and the plasma-membrane (6). This was established using a purified lyase of Erwinia chrysanthemi.

Subsequent work has led to the partial characterization of the regulatory mechanism governing trans-eliminase synthesis in isolate 14 (22). As had been reported for other isolates, the lyase synthesis was inducible and subject to nonspecific catabolite repression (22, 65). It was also shown in isolate 14 that lyase production was under cyclic adenosine-3',5'-monophosphate (cAMP) control. This is neither surprising nor novel in light of available information for comparable enzyme systems in Escherichia coli (51). Follow up work has led to the isolation and partial characterization of the inducer in this suspected lac operon type system (24). At present it appears that the inducer is an unsaturated digalacturonic acid.

Further investigation of the control mechanism for trans-eliminase synthesis in isolate 14 led to the induction and isolation of cAMP deficient mutants (40). Except for a low constitutive level of lyase activity, these mutants were unable to synthesize lyase even in the presence of the inducer. Only when cyclic AMP was supplied with the inducer could lyase synthesis resume.

For the majority of this work with isolate 14, the interpretations were in part based upon the assumption that there was one intracellular polygalacturonic acid trans-eliminase. A more intensive investigation and characterization of the intracellular trans-eliminase may fortify the interpretations of previous work. It may also establish the presence or absence of an oligogalacturonic acid trans-eliminase as reported for isolate 157 of Erwinia carotovora.

## MATERIALS AND METHODS

Culturing of Erwinia carotovora. All experiments were conducted using Erwinia carotovora (Jones) Holland, isolate 14 which is auxotrophic for threonine (EC 14 thr) (40). EC 14 thr was maintained on minimal salts (per liter; 1.5 g  $\text{KH}_2\text{PO}_4$ , 7.15 g  $\text{Na}_2\text{HPO}_4$ , 3.0 g  $(\text{NH}_4)_2\text{SO}_4$ , 0.02%  $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ ) nutrient agar slants supplemented with 0.5% yeast extract, 1.0% casein hydrolysate, 0.5% glucose, plus 0.02% threonine. After slants were inoculated, the culture was incubated for 24-48 hours at 30 C, then stored at 4 C. Subculturing was performed every two to four weeks.

Periodically these cultures were checked for their threonine auxotrophy, ability to degrade sodium polypectate (NaPP) (Sigma Chemical Co., Box 14508, St. Louis, MO 63118), and their capacity to induce soft rotting of potato tissue. The requirement of threonine for growth was tested by subculturing EC 14 thr onto glucose minimal salts petri plates plus or minus the threonine supplement. The ability to degrade NaPP was monitored by streaking EC 14 thr onto NaPP, yeast extract and minimal salts agar plus threonine. After 24-48 hours the streaked plate was flooded with 1.0% hexadecyltrimethyl ammonium bromide (TAB) (Eastman Kodak Co., Rochester, NY 14650) (20). A zone of clearing surrounding the colonies was evidence of NaPP degradation. Testing for the capacity to induce soft rot disease was accomplished by inoculating surface sterilized potato tubers with log phase EC 14 thr and incubating 7-10 days at room temperature. The inoculation procedure consisted of removing a plug of tuber tissue

with a number 8-10 cork borer and excising a central 3-5 cm section of the plug. One end was placed back into the hole and sealed with lanolin. After adding 1-2 ml of the bacterial suspension to the well, the other end of the plug was replaced and sealed with lanolin. The tuber was then placed in a plastic bag with a moist paper towel, sealed and incubated.

Liquid culture media contained 50 ml of a minimal salts medium supplemented with 0.02% threonine and 1.0% NaPP and were inoculated with EC 14 thr from a slant. A side arm Erlenmeyer flask containing the culture was incubated for 8-12 hours (or overnight) on a shaker water bath (150 revs per minute at 30 C) and was used as inoculum for larger cultures (500 ml in a 2000 ml Erlenmeyer flask) containing the same medium. These cultures were then placed on a shaker at room temperature (25-27 C) and incubated until the bacteria reached the desired population density as measured in a Bausch and Lomb Spectronic 20.

Enzyme extraction and purification. Cells from liquid shake cultures were harvested during mid to late log phase (O.D.<sub>600</sub> 0.6-0.8) and were collected by centrifugation (6,000g @ 4 C for 20 min) using a GSA rotor in a Sorvall RC2-B refrigerated centrifuge. The bacteria were resuspended in 0.05 M Tris-HCl buffer (pH 8.0) and re-centrifuged to wash away any extracellular enzyme still adsorbed to the cells. The pelleted cells were resuspended in 1-2 ml of the same buffer and transferred to small vials in an ice bath. The bacteria were then lysed by ultrasonication with a Branson Sonifier Cell Dis-

rupter microtip at 30W for 3 minutes. The cell sonicate was centrifuged (20,000g @ 4 C for 20 min) to pellet the cellular debris and the supernatant was saved for further purification of the intracellular pectic enzyme(s).

The crude enzyme preparation was gradually brought to 50% saturation with ammonium sulfate, allowed to stand at 4 C for 30 minutes, centrifuged (20,000g @ 4 C for 30 min), and the precipitate discarded. The resulting supernatant was then gradually brought to 95% saturation with ammonium sulfate and again allowed to stand at 4 C for 30 minutes, centrifuged (20,000g @ 4 C for 30 min) and the supernatant decanted. The precipitate was resuspended in 1-2 ml of distilled water (dH<sub>2</sub>O) and dialysed overnight at 4 C against several liters of dH<sub>2</sub>O. The dialysate (3 ml) was applied to 2.5 X 25 cm diethylaminoethyl (DEAE) cellulose column at 4 C and eluted according to the method of Mount et al (39). Next, 30 ml of 0.05 M Tris-HCl buffer, pH 8.0, was added, followed by a stepwise gradient of 50 ml portions of the same buffer containing increasing concentrations (0.05 N, 0.1 N, 0.2 N, 0.3 N and 0.4 N) of sodium chloride (NaCl). Five ml fractions were collected and assayed for enzyme activity.

The DEAE fractions showing trans-eliminase activity which eluted with the void volume, were pooled and subjected to isoelectric focusing in an LKB 8101 Ampholine electrofocusing apparatus equipped with a 110 ml column (LKB-Producter AB, Bromma, Sweden) and an LKB 3371 D DC power source. The column contained an ampholine carrier with a range of pH 3-10 (39). Focusing was conducted by the procedure of Mount et al (39) modified to the extent that electrofocusing was carried

out at 4 C for 72-96 hours until the voltage had stabilized. As five ml fractions were being collected, the pH of each fraction was measured immediately. These fractions were dialysed overnight at 4 C against several liters of dH<sub>2</sub>O and analysed for trans-eliminase activity.

Disc gel electrophoresis was carried out using the method of Weber and Osborn (63) as modified by Leammli (30). All enzyme samples were isoelectrically focused prior to application to the polyacrylamide gels. Electrophoresis continued for 3-4 hours at 1-2 milliamps per gel and was stopped when the tracking dye neared the end of the gel. Gels were fixed and stained in a solution containing 0.125 g of Coumassie brilliant blue, 454 ml of 50% methanol and 46 ml of glacial acetic acid. The gels were destained in an aqueous solution of methanol and acetic acid as described by Weber and Osborn (63).

Enzyme assays. Pectic enzyme activity was measured by the periodate-thiobarbituric acid assay (TBA) (52,64), the Nelsons reducing group analysis (47), and/or the viscometric assay (9,11). For screening of enzyme activity the substrate was comprised of 1.2% NaPP in 0.05 M Tris-HCl buffer at pH 8.5 containing  $2 \times 10^{-4}$  Ca<sup>++</sup> (CaCl<sub>2</sub>). In the TBA assay 100  $\mu$ l of enzyme solution were incubated at 30 C for one hour or varying times with 100  $\mu$ l of substrate. An optical density of 0.3 indicated the formation of 0.1  $\mu$ mole of unsaturated product. A unit of activity was expressed as the amount of enzyme yielding an increase in absorbance of 0.1 in 1.0 hour, or the amount of enzyme yielding .01  $\mu$ mole of unsaturated product in one hour. Specific activity was

expressed as units of activity per milligram of protein per ml of enzyme solution. For the reducing group analysis of reducing sugars released from NaPP, a standard curve was prepared using D-galacturonic acid in varying concentrations (0-400  $\mu\text{g}$ ) and absorbance was measured at 500 nm. Activity was expressed as micromoles of product formed per milligram of protein per ml of enzyme solution. In the viscometric assay, 5.0 ml of substrate plus one ml of enzyme solution were added to a Ostwald-Fenske 300 viscometer and depolymerization of NaPP was measured over time. Relative activity was expressed as the reciprocal of the time in minutes for 50% loss in viscosity times  $10^3$  (9,11). Specific activity is the relative activity per milligram of protein per ml of enzyme solution. Autoclaved enzyme and/or  $\text{dH}_2\text{O}$  were used as the controls.

Characterization. For determination of pH optimum of enzyme activity the TBA assay or the Nelsons reducing group analysis were used utilizing four buffer systems: 0.05 M acetate buffer (pH 4.5-6.5), 0.05 M phosphate buffer (pH 6.0-7.5), 0.05 M Tris-HCl buffer (pH 7.0-9.0), and 0.05 M NaOH-glycine buffer (pH 9.0-10.0). Enzyme-substrate reaction mixtures were incubated at 30 C for one hour or for various time intervals.

To determine ion dependence for enzyme activity, NaPP (1.2%) was dissolved in 0.05 M Tris-HCl buffer pH 8.5, followed by the addition of ethylenediamine tetraacetic acid (EDTA) (Sigma) to a final concentration of 0.5 mM in order to chelate endogenous ions. Then  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$ , or  $\text{Mn}^{++}$  were added to a final concentration of 1.0 mM. These

substrates were used with the TBA, reducing group, and viscometric assays for the determination of ionic requirements. These specific substrates were also used for all further characterization studies.

Identification of reaction products. Separation and identification of reaction products were attempted using descending paper chromatographic procedures described in the literature (37,53). After incubation of reaction mixtures at 30 C for varying times, 50  $\mu$ l samples were applied to Whatman paper No. 4. The solvent system employed was a mixture of pyridine-ethyl acetate-acetic acid-water (5:5:1:3). The chromatograms were developed with saturated silver nitrate in acetone and 0.5 N NaOH in ethanol.

The chain length of the enzymatic breakdown products of NaPP was determined by two methods. The first method was the comparison of the products rate of migration on paper chromatograms in comparison to the standard D-galacturonic acid ( $R_{gal}$  value). The  $R_{gal}$  values for the breakdown products were compared to those reported in the literature using known chain lengths (15,16,35,37,44,45,46). The ratio of uronic acid units as measured by the carbazole analysis (14) to free reducing groups (47) was also used to calculate the chain length of the reaction products.

Immunological comparison. Immunological comparison studies were conducted using antibody prepared against the purified (isoelectric focused) extracellular PGTE. Purified PGTE was injected into New Zealand white rabbits in a series of three, two-milligram injections on a six week schedule. Titre of the antiserum to endo PGTE obtained

from the rabbits was 1:360 as determined by microprecipitin-ring tests (23). The antisera was placed in the center well of an Ouchterlony (50) double diffusion petri plate and fractions to be tested were placed in the exterior wells. Incubation was carried out at room temperature until precipitate bands appeared (24-72 hours).

Protein concentrations were determined using the method of Lowry et al (32). Crystalline bovine serum albumin was used as the reference protein.

All chemicals used in this investigation were purchased from Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178, unless otherwise stated.

## RESULTS

After 48 hours incubation at 30 C, growth of EC 14 thr consistently occurred only on threonine supplemented medium. An approximately one centimeter zone of clearing appeared repeatedly around streak colonies of EC 14 thr after flooding the sodium polypectate (NaPP)- minimal salts plate with hexadecyltrimethyl ammonium bromide. Within 7-10 days following inoculation, potato tubers exhibited typical soft rot symptoms resulting in tuber disintegration. These tests demonstrated the stability of this organisms' characteristics and insured the confidence that all experiments were conducted using the same specific strain of Erwinia carotovora. For the sake of consistency, reproducibility, and the integration of these results with prior work on this organism, the bacterial cells for enzyme extraction were always harvested during mid to late log phase.

The isolation and purification schematic of the intracellular enzymes used in this study (Fig. 1) was comparable to that used for the isolation and purification of the extracellular PGTE (39). The degree of purification with each step in the procedure was also comparable. After DEAE cellulose column chromatography, two TBA positive peaks appeared (Fig. 2). The first peak showed high activity and eluted with the column void volume. The second peak exhibited low to moderate activity and was eluted with 0.2-0.3 N NaCl. When DEAE fraction 1 was subjected to isoelectric focusing in an ampholine range of pH 3-10, 3 TBA positive fractions resulted. These three PGTEs had

Fig. 1. Scheme for the isolation and purification of the polygalacturonate trans-eliminases of Erwinia carotovora (Jones) Holland isolate 14.

PURIFICATION OF THE INTRACELLULAR PECTIC  
ENZYME COMPLEX IN EC14 thr

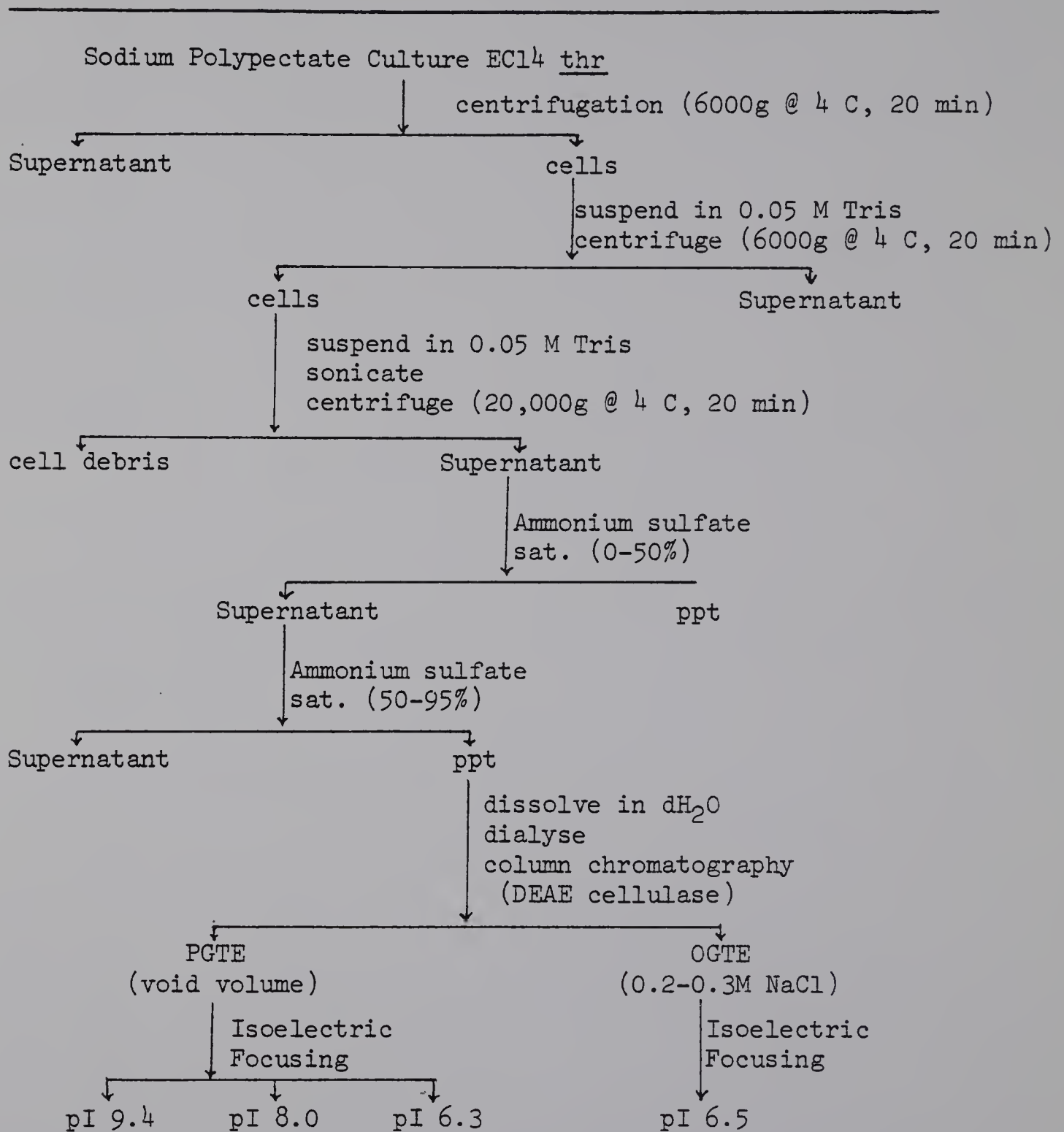
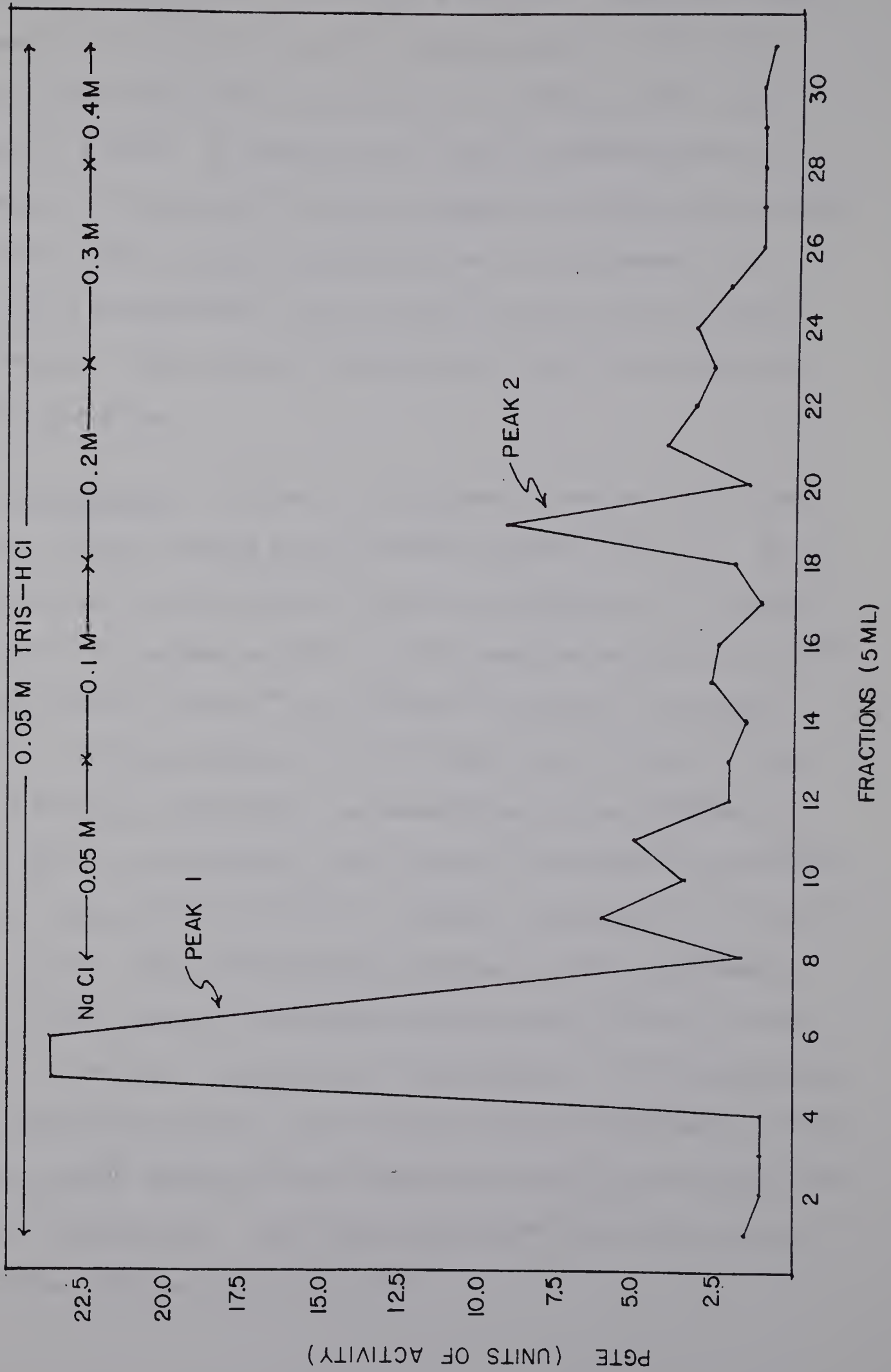


Fig. 2. Separation of polygalacturonate acid trans-eliminases by DEAE cellulose column chromatography. Three ml of dialyzed enzyme from 50-95% ammonium sulfate saturation of crude cell sonicate of Erwinia carotovora were applied to a DEAE cellulose column (2.5 X 25 cm) at 4 C. Five ml fractions were collected and enzyme activity analyzed by the periodate-thiobarbituric acid assay (64).



isoelectric points of 9.4, 8.0, and 6.3 (Fig. 3). Henceforth these enzymes will be referred to as PDI (intracellular 9.4 PGTE), PDIIa (extracellular PGTE), PDII (intracellular 8.0 PGTE), PDIII (intracellular 6.3 PGTE). PD signifies the class of enzymes pectin depolymerases. Each enzyme fraction was applied to three concentrations (7.5%, 10%, 12%) of polyacrylamide gels and electrophoresed. On each of the concentrations only one band from each enzyme fraction was detected. This indicated the presence of just one protein per enzyme preparation.

Ion Dependence. Testing for dependence of enzyme activity upon divalent cations revealed three different patterns (Table 1). As is the case with the extracellular PDIIa, the intracellular PDI required 1.0 mM  $\text{Ca}^{++}$  for optimal activity. PDII showed low activity with  $\text{Ca}^{++}$ , moderate activity with  $\text{Mg}^{++}$ , but required 1.0 mM  $\text{Mn}^{++}$  for optimal activity. With the addition of 0.5 mM EDTA, the activities of both PDI and PDII were eliminated. As demonstrated by the addition of 0.5 mM EDTA to the substrate, PDIII showed no requirement for divalent cations. The activity of PDIII was, however, increased by 1.0 mM  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$ , or  $\text{Mn}^{++}$ . These differential responses to cation supplements seem to offer a means of separately monitoring the activity of each enzyme in a mixture containing all three (Table 2). To date this has been impossible since the routine assays used for measurement of PGTE activity cannot distinguish each enzyme from the others when they are assayed simultaneously. All three enzymes form unsaturated products and release reducing sugars from NaPP.

Fig. 3. Isoelectric focusing of peak 1 polygalacturonate trans-eliminase from DEAE cellulose column chromatography (Fig. 2). Focusing was conducted using ampholine carriers with a pH range of 3-10 at 4 C for 72-96 hours until the current had stabilized. Five ml fractions were collected and the pH measured immediately. After overnight dialyzation at 4 C, each fraction was assayed for enzyme activity. The pI values for the enzymes are 9.4, 8.0, and 6.3.

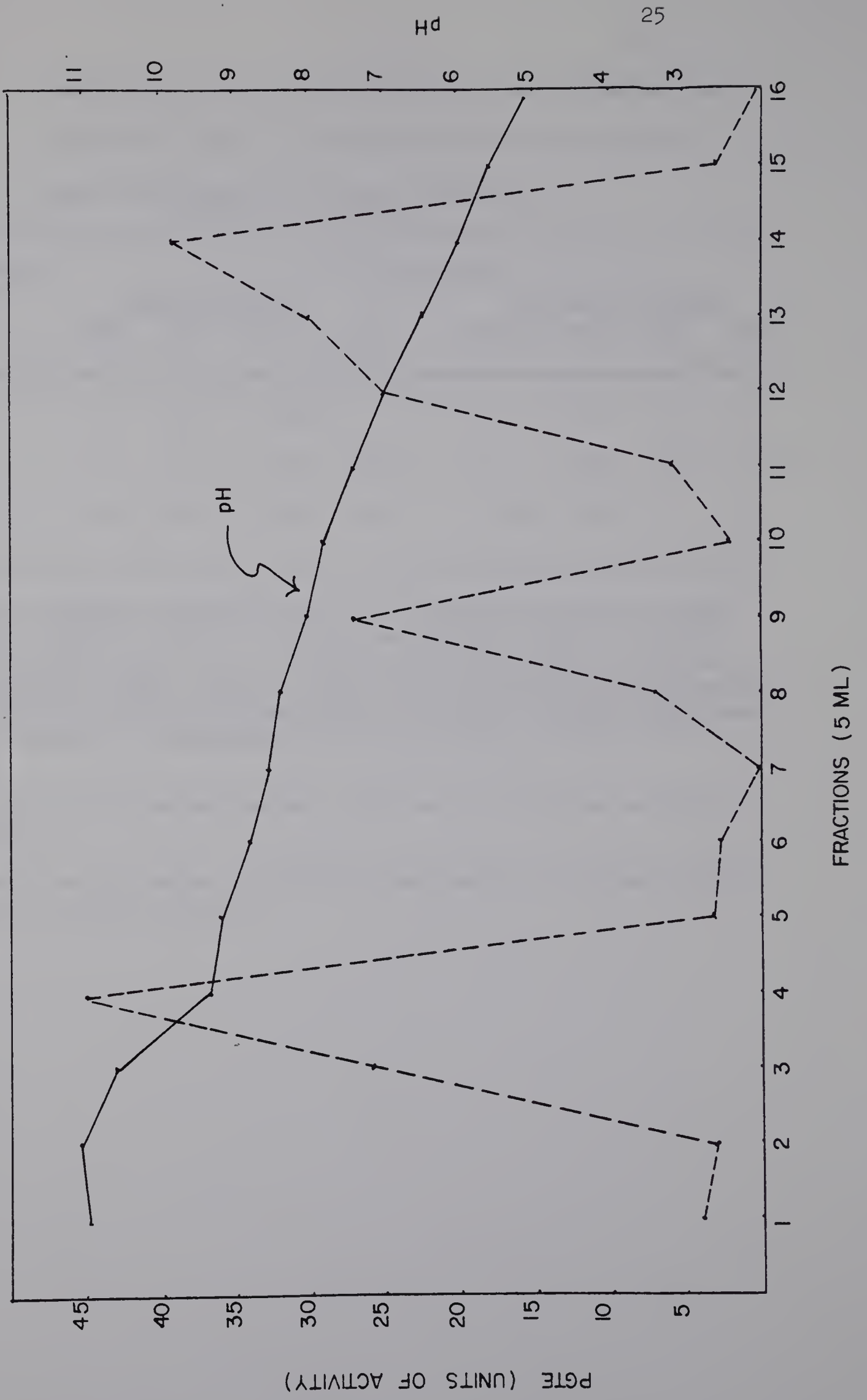


Table 1. Dependence upon divalent cations of the trans-eliminase activity of three intracellular pectic acid lyase enzymes from Erwinia carotovora isolate 14.

Isoelectric Focused PGTE	PGTE Activity <sup>a</sup>							
	<u>μMoles Product</u> <sup>b</sup>				<u>Specific Activity</u> <sup>c</sup>			
	EDTA	Ca <sup>++</sup>	Mn <sup>++</sup>	Mg <sup>++</sup>	EDTA	Ca <sup>++</sup>	Mn <sup>++</sup>	Mg <sup>++</sup>
PDI	0	0.05	0.003	0	0	7.1	0.43	0
PDII	0	0.01	0.06	0.015	0	0.06	0.65	0.16
PDIII	0.02	0.023	0.022	0.023	0.07	0.097	0.1	0.1

<sup>a</sup>Reaction mixtures contained 100 μl of isoelectric focused enzyme solution plus 100 μl of 1.2% sodium polypectate (NaPP) in 0.05 M Tris-HCl buffer at pH 8.5. Prior to incubation with the enzyme, the substrate (NaPP) had been treated with ethylenediaminetetracetic acid (EDTA) to a final concentration of 0.5 mM. This was then used unsupplemented or supplemented with a specific ion (Ca<sup>++</sup>, Mn<sup>++</sup> or Mg<sup>++</sup>).

<sup>b</sup>Expressed as the μmoles product formed per ml of enzyme solution per hour.

<sup>c</sup>Expressed as the μmoles of product formed per ml of enzyme solution per hour per mg of protein.

Table 2. Separation of trans-eliminase activities of three pectin lyases from Erwinia carotovora based upon their ion requirements.

Isoelectric Focused PGTE	Enzyme Activity on Specific Assay Medium <sup>a</sup>		
	EDTA+Ca <sup>++</sup>	EDTA+Mg <sup>++</sup>	EDTA
PDI	+ <sup>b</sup>	- <sup>c</sup>	-
PDII	+	+	-
PDIII	+	+	+

<sup>a</sup>Reaction mixtures contained 100  $\mu$ l of isoelectric focused enzyme solution plus 100  $\mu$ l of 1.2% sodium polypectate (NaPP) in 0.05 M Tris-HCl buffer at pH 8.5. Prior to incubation with the enzyme, the substrate (NaPP) had been treated with ethylenediamine tetraacetic acid (EDTA) to a final concentration of 0.5 mM. This was then used unsupplemented or supplemented with a specific ion (Ca<sup>++</sup>, Mn<sup>++</sup>, or Mg<sup>++</sup>) to a final concentration of 1.0 mM.

<sup>b</sup>Measurable activity (Table 1)

<sup>c</sup>No detectable activity (Table 1)

Characteristics of PDI. The PGTE with an isoelectric point of 9.4 closely corresponded with the isoelectric point of the extracellular PGTE (pI 9.4) (61). As was demonstrated with PDIA, PDI exhibited optimal activity at pH 8.0-8.5 in the presence of 1.0 mM  $\text{Ca}^{++}$  (Fig. 4). Rapid decrease in a solution of NaPP (Table 3) as well as the yielding of several size reaction products shown on paper chromatograms (Fig. 5A), indicated an endo or random mode of depolymerization of NaPP. These reaction products were unsaturated as shown by the TBA assay (Table 4).

At pH 6.0 in the presence of 1.0 mM  $\text{Ca}^{++}$  or  $\text{Mn}^{++}$ , PDI produced several sized reaction products shown on paper chromatograms (Fig. 5A). These reaction products, however, were saturated as demonstrated by the TBA and reducing group analyses (Table 4). Isoelectric focused PDIA was similarly tested and the same results obtained. At pH 6.0 in the presence of  $\text{Ca}^{++}$  (or  $\text{Mn}^{++}$  - data not shown) or no ion supplement, the enzyme substrate reaction mixture was positive for reducing sugars and negative for unsaturated bonds (Fig. 4), and showed several size reaction products on paper chromatograms. These data strongly suggest that PDI and the solo extracellular PGTE, PDIA, are the same protein or two forms of the same protein. Dependent upon the conditions, this protein has the capacity to depolymerize NaPP by two different mechanisms. Under alkaline conditions (pH 8.0-9.0) in the presence of  $\text{Ca}^{++}$  it cleaves NaPP in a trans-eliminase or lytic manner, yet under acid conditions (pH 5.0-6.0) in the presence or absence of divalent cations it cleaves NaPP hydrolytically.

Fig. 4. The activity of PDI over the pH range of 4.5-9.0.

Isoelectric focused purified enzyme was assayed for the ability to form unsaturated oligouronides in the presence (X—) or absence (X---) of calcium. PDI was also assayed for the ability to release free reducing groups from substrate in the presence (o—) or absence (o---) of calcium. Enzyme-substrate reaction mixtures contained 100  $\mu$ l enzyme solution plus 100  $\mu$ l of 1.2% sodium polypectate (0.5 mM with respect to EDTA or 1.0 mM with respect to calcium). Incubation was for 1.0 hour. The following buffer systems were used: 0.05 M acetate buffer (pH 4.5-6.5), 0.05 M phosphate buffer (pH 6.0-7.5), 0.05 M Tris-HCl buffer (pH 7.0-9.0), and 0.05 M NaOH-glycine buffer (pH 9.0-10.0).

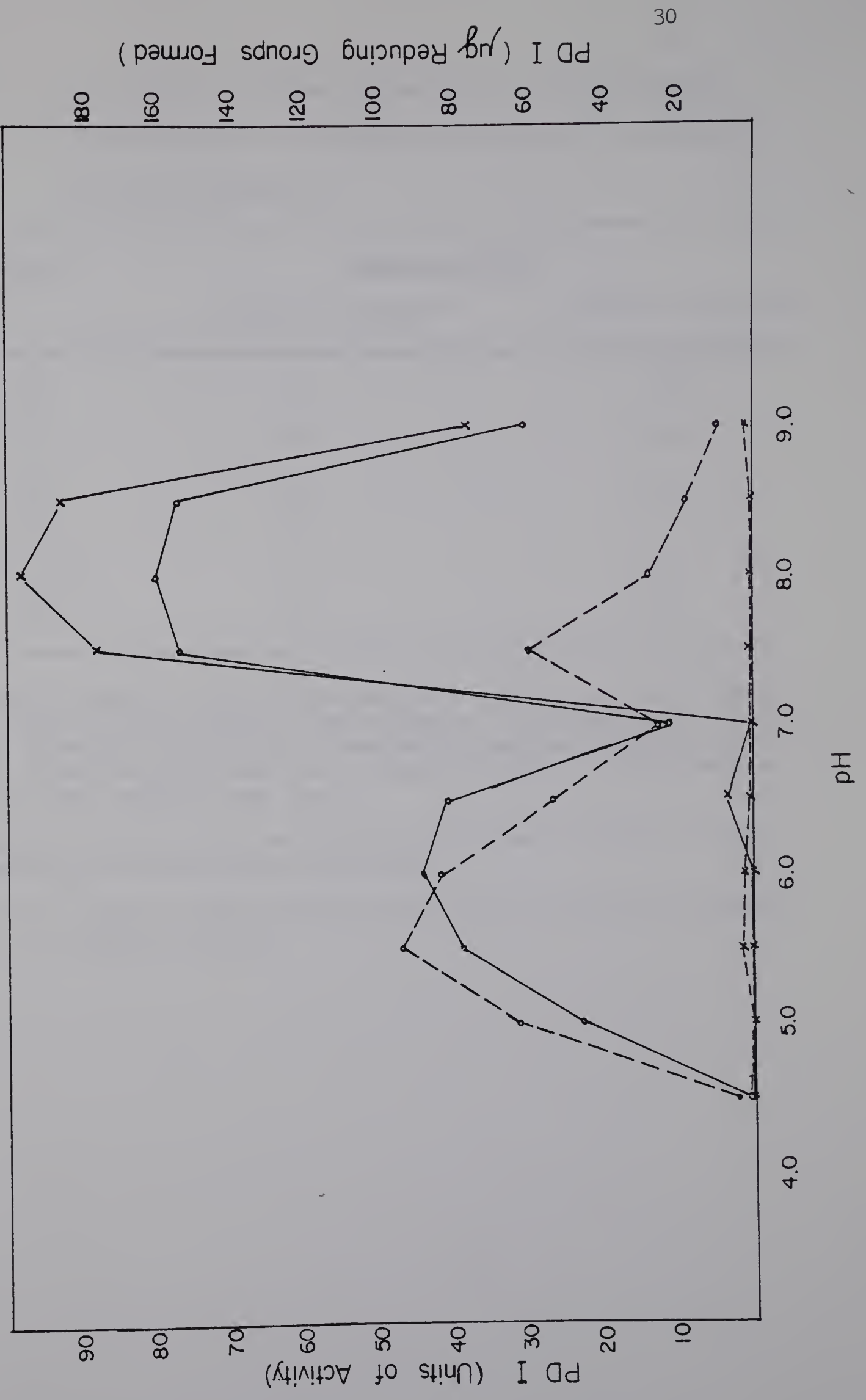


Table 3. The abilities of three intracellular polygalacturonate trans-eliminases to decrease the viscosity of a solution of sodium polypectate.<sup>a</sup>

Isoelectric Focused PGTE	<u>Enzyme Activity</u>	
	Relative Activity <sup>b</sup>	Specific Activity <sup>c</sup>
PDI	Ca <sup>++</sup>	71
	Mn <sup>++</sup>	<1.0
PDII	Ca <sup>++</sup>	<1.0
	Mn <sup>++</sup>	58
PDIII	Ca <sup>++</sup>	<1.0
	Mn <sup>++</sup>	<1.0

<sup>a</sup>Substrate comprised 1.2% sodium polypectate (NaPP) in 0.05 M Tris-HCl buffer at pH 8.5 plus 1.0 mM calcium or 1.0 mM manganese. Five ml of substrate plus 1.0 ml of isoelectric purified enzyme were added to an Ostwald-Fenske 300 viscometer, mixed, and depolymerization of NaPP measured over time.

<sup>b</sup>Relative activity is the reciprocal of the time in minutes to 50% reduction in viscosity times 10<sup>3</sup> (9,11).

<sup>c</sup>Specific activity is the relative activity per milligram of protein per ml of enzyme solution.

Fig. 5. Schematic representation of chromatograms of reaction mixture products produced by various isoelectric focused polygalacturonate trans-eliminases. Reaction mixtures were incubated at 30 C for 10 hours. The solvent system employed was pyridine-ethylacetate-acetic acid-water (5:5:1:3). Reaction products were chromatographed on Whatman paper No. 4 at room temperature for 10 hours. A silver nitrate saturated acetone solution was used to develop the chromatograms. AI = PDI + NaPP at pH 6.0  $\pm$  Ca<sup>++</sup>; AII = PDI + NaPP at pH 8.5 + Ca<sup>++</sup>; AIII = D-galacturonic acid standard. BI = PDII + NaPP at pH 6.0 + Mn<sup>++</sup>; BII = PDII + NaPP at pH 7.5 + Mn<sup>++</sup>; BIII = PDII + NaPP at pH 7.5 + Ca<sup>++</sup>; BIV = PDII + NaPP at pH 8.5 + Mn<sup>++</sup>; BV = PDII + NaPP at pH 8.5 + Ca<sup>++</sup>. CI = PDIII + NaPP at pH 6.0 + Mn<sup>++</sup>; CII = PDIII + NaPP at pH 7.5  $\pm$  Mn<sup>++</sup>; CIII = PDIII + NaPP at pH 8.5  $\pm$  Mn<sup>++</sup>; CIV = D-galacturonic acid standard; CV = acid hydrolysed product of PDIII on NaPP at pH 8.5 + Mn<sup>++</sup> (acid hydrolysis was carried out with 1.0 N HCl in boiling water bath for 0.5 hours). DI = D-galacturonic acid standard; DII = PDIII + NaPP at pH 8.5 + Mn<sup>++</sup>; DIII = PDIV + product of PDIII (unsaturated digalacturonic acid) at pH 7.5  $\pm$  Ca<sup>++</sup>; DIV = PDIV + product of PDIII at pH 8.5  $\pm$  Ca<sup>++</sup>.

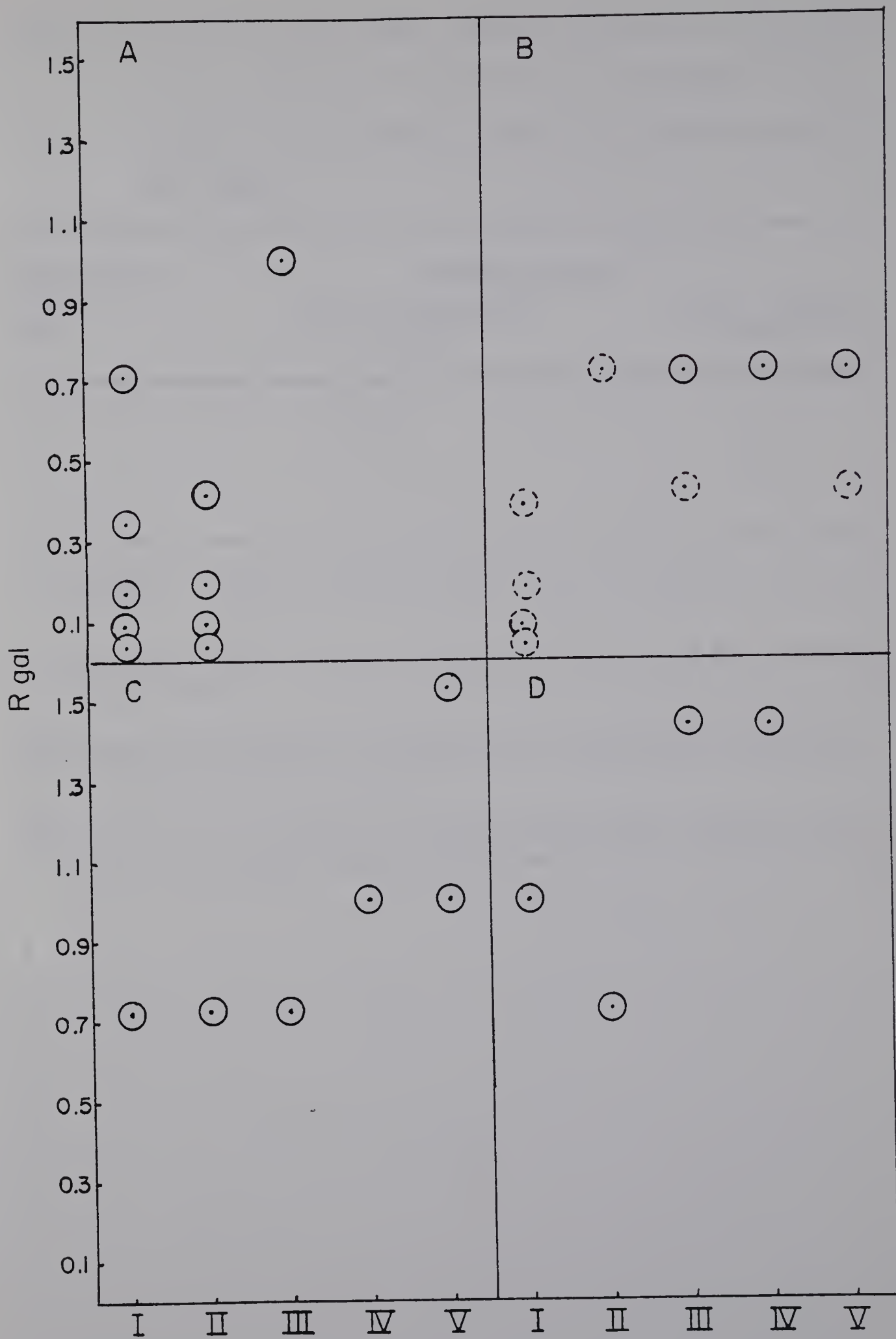


Table 4. Activity of PDI under various conditions as indicated by the formation of unsaturated oligouronides or the release of free reducing sugars from sodium polypectate (NaPP).

Isoelectric Focused PDI	<u>Enzyme Activity</u>	
	$\mu$ moles unsaturated product <sup>a</sup>	$\mu$ moles reducing product <sup>b</sup>
A	3.20	3.25
B	0	3.15

A = PDI+NaPP (1.2% in 0.05 M Tris-HCl buffer) at pH 8.5 + 1.0 mM Ca<sup>++</sup>

B = PDI+ NaPP (1.2% in 0.05 M acetate buffer) at pH 6.0 + 1.0 mM Ca<sup>++</sup> or Mn<sup>++</sup>

<sup>a</sup>Expressed as the  $\mu$ moles of unsaturated oligouronide formed per ml of enzyme solution per hour.

<sup>b</sup>Expressed as the  $\mu$ moles of free reducing sugars released from NaPP per ml of enzyme solution per hour.

Characteristics of PDII. The PGTE with an isoelectric point of 8.0 exhibited activity from pH 5.5-10.0 with optimal activity at pH 8.5 in the presence of 1.0 mM  $Mn^{++}$ . Supplemented with 1.0 mM  $Ca^{++}$  or  $Mn^{++}$  PDII decreased the viscosity of a solution of NaPP than PDI. Though the relative activities were comparable at pH 8.5 when the PDI reaction mixture was supplemented with  $Ca^{++}$  and the PDII reaction mixture was supplemented with  $Mn^{++}$ , the specific activities revealed a 16 fold difference (Table 3). After applying these reaction mixtures to paper chromatograms it was evident that the reaction products of PDII differed greatly from PDI reaction products. Whereas PDI gave an array of reaction products, PDII yielded only two products (Fig. 5B). The main product of the PDII reaction migrated much faster ( $R_{gal}$  0.72) than any of the PDI products. The chromatograms indicated an exo or terminal mode of attack during depolymerization of NaPP by PDII, however, PDII did show some endo like properties as evidenced by the viscometric data and the fact that the products of a PDII reaction at pH 6.0 showed trace amounts of products similar to those obtained in a PDI reaction mixture (Fig. 5 A,B).

Characteristics of PDIII. The pH profile for activity of PDIII is similar to that of PDII in the presence of 1.0 mM  $Mn^{++}$ ,  $Ca^{++}$ , or  $Mg^{++}$ . Irrespective of the conditions PDIII decreased the viscosity of a solution of NaPP very slowly compared to either PDI or PDII (Table 3). When the PDIII reaction mixture was applied to paper chromatograms only one reaction product was observed. This one product had the same  $R_{gal}$  value (0.72) as the fast migrating spot produced

by PDII (Fig. 5C). These results clearly showed an exo mode of depolymerization of NaPP by PDIII.

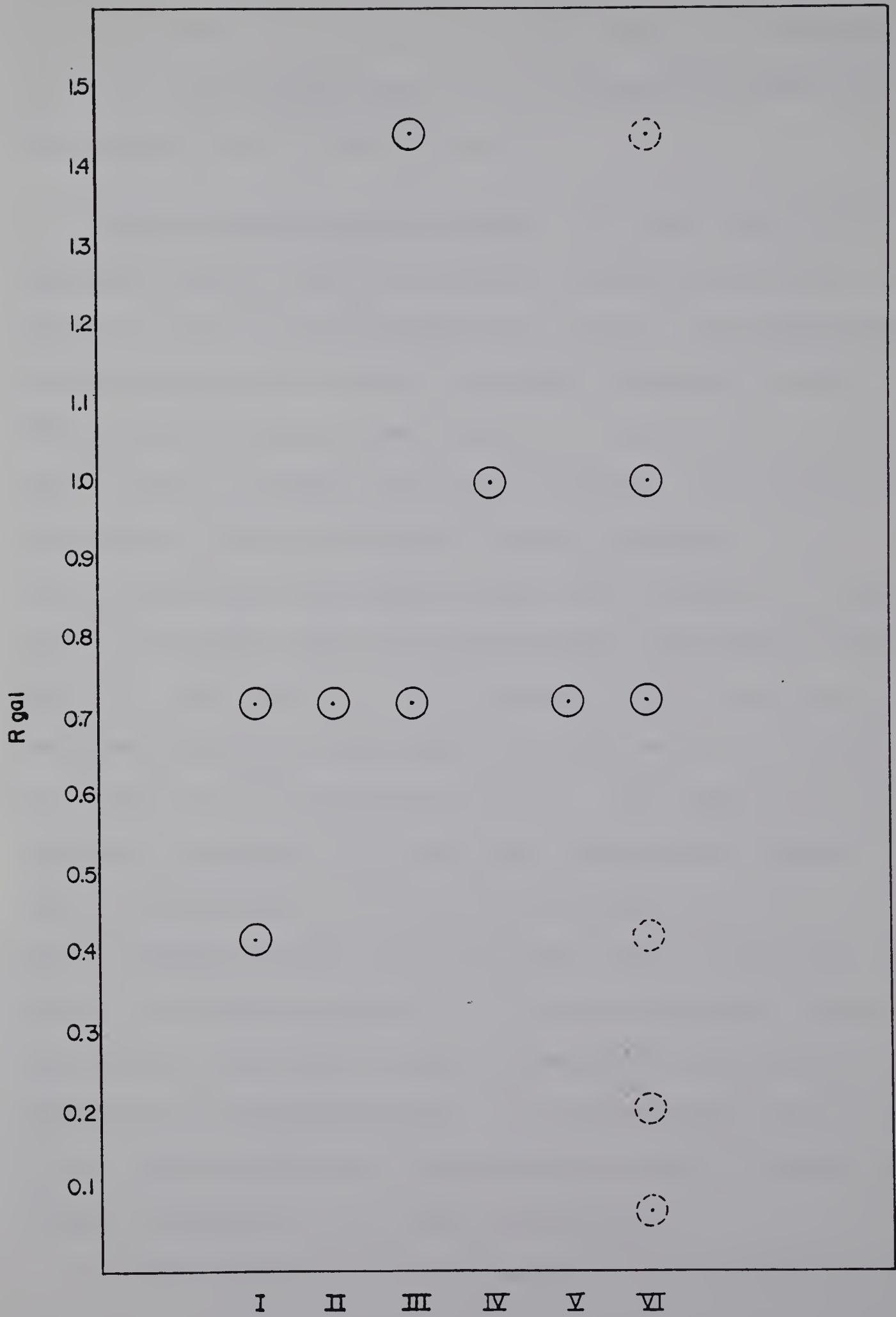
Characterization of PDIV. When DEAE peak two was subjected to isoelectric focusing in an ampholine range of pH 3-10, one TBA positive fraction resulted. This enzyme, designated as PDIV, had an isoelectric point of pH 6.5. Unlike the other pectin depolymerases reported here, PDIV was very unstable if not lyophilized and kept at -20 C. After focusing, PDIV lost approximately 70% of its activity as compared to that eluted from the DEAE column. Since the isoelectric focusing of DEAE fraction two yielded only one pectic enzyme, all subsequent experiments employed DEAE partially purified PDIV in order to preserve its activity.

PDIV exhibited a limited capacity for depolymerization of NaPP. When NaPP was used as the substrate, PDIV showed its best activity at pH 7.5 in the presence of 1.0 mM  $\text{Ca}^{++}$ . This reaction mixture was applied to paper chromatograms (Fig. 6) and small amounts of two products appeared ( $R_{\text{gal}}$  0.4 and 0.72). These were the same two products yielded by PDII, indicating an exo mode of depolymerization of NaPP by PDIV under these conditions. PDIV required  $\text{Ca}^{++}$  or  $\text{Mn}^{++}$  to exhibit its exo activity.

In order to determine if this enzyme had oligogalacturonate transeliminase properties similar to that observed by Moran et al (38), PDIV was reacted with the unsaturated dimer produced by PDIII in a NaPP reaction mixture. PDIV converted the unsaturated dimer into one product ( $R_{\text{gal}}$  1.4) which migrated ahead of the D-galacturonic acid standard

Fig. 6. Schematic representation of a chromatogram of reaction mixture products produced by various polygalacturonate isoelectric focused trans-eliminases. Reaction mixtures were incubated at 30 C for 10 hours. The solvent system used was pyridine-ethylacetate-acetic acid-water (5:5:1:3). Reactions were chromatographed on Whatman paper No. 4 at room temperature for 10 hours. A silver nitrate saturated acetone solution was used to develop the chromatograms.

I = PDIV + NaPP at pH 7.5 +  $\text{Ca}^{++}$ ; II = PDI, PDII, PDIII + NaPP at pH 8.5 +  $\text{Ca}^{++}$  +  $\text{Mn}^{++}$ ; III = PDI, PDII, PDIII, PDIV + NaPP at pH 8.5 +  $\text{Ca}^{++}$  +  $\text{Mn}^{++}$ ; IV = D-galacturonic acid standard; V = PDIII + NaPP at pH 8.5 +  $\text{Mn}^{++}$ ; VI = PDI, PDIV + NaPP at pH 6.0 +  $\text{Ca}^{++}$  +  $\text{Mn}^{++}$ .



(Fig. 5D). This product was TBA positive and gave a ratio of one uronic acid unit to one free reducing group (Table 5). The ability of PDIV to convert unsaturated dimer into unsaturated monomer was demonstrated at pH 7.5 and 8.5 (Fig. 5D).

Identification of reaction products. The chain length of the enzymatic breakdown products of NaPP was determined for all the enzymes. At pH 8.5 in the presence of 1.0 mM  $\text{Ca}^{++}$ , PDI gave products ranging from unsaturated dimer to suspected unsaturated pentamer as indicated by their  $R_{\text{gal}}$  values (15,37,46). At pH 6.0 PDI also produced a range of products from dimer to pentamer. PDII produced a small amount of digalacturonic acid but the predominant product migrated much faster than digalacturonic acid according to its  $R_{\text{gal}}$  value. This faster spot was the same and only spot yielded by PDIII (Fig. 5C). When the ratio of free reducing groups to uronic acid units was determined on this product it gave a ratio of two uronic acid units to one reducing group (Table 5). This product was also unsaturated as indicated by the TBA assay. These results suggest that the fast migrating spot is an unsaturated digalacturonic acid. The smallest reaction product of PDI also gave a ratio of two uronic acid units to one reducing group (24). It too was unsaturated. Though they migrated differently on paper chromatograms, biochemically they both key out to unsaturated dimer. It is possible that they are isomers of each other or differ in a functional group (acetyl or methyl) which affects the migration rate on paper chromatograms.

The fast migrating product of the PDIII reaction mixture was acid

Table 5. Determination of chain length of reaction products from incubation of trans-eliminases of Erwinia carotovora with specific substrates.

	$R_{gal}^a$	COOH/CHO <sup>b</sup>	Unsaturated product formed <sup>c</sup>	Uronic acid residues/chain
A	0.72	1.9:1.0	+	2
B	1.42	0.97:1.0	+	1
C	1.42	0.97:1.0	+	1

A = Product formed upon reaction of PDIII with sodium polypectate (NaPP) at pH 8.5 + 1.0 mM Mn<sup>++</sup>

B = Fast migrating product formed upon reaction of PDI, PDII, PDIII and PDIV with NaPP at pH 8.5 + Ca<sup>++</sup> and Mn<sup>++</sup> (each 1.0 mM)

C = Product formed upon reaction of PDIV with the product formed by reaction of PDIII with NaPP at pH 8.5 + Mn<sup>++</sup> (A).

<sup>a</sup> $R_{gal}$  is the ratio of the distance migrated on a paper chromatogram of the unknown product to the distance migrated by the D-galacturonic acid standard. Paper chromatogram procedures are described in the text.

<sup>b</sup>Estimation of the uronic acid residues was determined by the carbazole reaction (14). Estimation of free reducing groups was determined by the Nelsons analysis (47).

<sup>c</sup>Detection of unsaturated product was accomplished using the periodate-thiobarbituric acid assay (64).

hydrolysed and applied to a paper chromatogram (Fig. 5C). This treatment of the unsaturated dimer yielded two spots; one corresponding to the D-galacturonic acid standard and one migrating far ahead of D-galacturonic acid. This fast moving spot resulting from acid hydrolysis corresponds to what has been reported in the literature as 5-formyl-3-furancarboxylic acid (16). This compound is a direct result of acid hydrolysis of the unsaturated dimer.

When a mixture of the various enzymes (PDIII, PDII, PDI, PDIV) were incubated for 10 hours with NaPP at pH 8.5 and the reaction products spotted on paper chromatograms two products appeared (Fig. 6). One spot migrated far ahead of D-galacturonic acid but slightly behind the fast migrating spot resulting from acid hydrolysis of the unsaturated dimer. This fast migrating spot gave a ratio of one uronic acid unit to one reducing group when subjected to the Nelsons-Carbazole tests (Table 5). The product also gave a very strong TBA reaction indicating unsaturation. Therefore it seems that the ultimate product from the action of all the enzymes working in conjunction with each other at this pH is the unsaturated monomer.

When all the enzymes were mixed and incubated with NaPP at pH 6.0, one additional product appeared that didn't result from the reaction of substrate with the isolated enzymes. This additional product co-migrated with the D-galacturonic acid standard. If purified PDI and PDIV were incubated with substrate at pH 6.0 several size reaction products resulted (Fig. 6), including D-galacturonic acid. The unsaturated monomer, which migrated ahead of the D-galacturonic acid standard, appeared only in trace amounts. Independently these enzymes showed

hydrolase activity in the absence of ions at pH 6.0 as determined by the TBA and Nelsons analyses (Tables 4 & 6 ). This suggests that a dual capacity for PDIV to act as a trans-eliminase or hydrolase depending upon the conditions as similarly reported for PDI and PDIA.

Immunological comparison. Each enzyme being studied was placed in an exterior well of an Ouchterlony double diffusion petri plate and an antisera prepared against purified PDIA was placed in the center well. After incubation at room temperature, only PDI cross reacted with the antibody of PDIA. This showed that PDII, PDIII, and PDIV were not antigenically related to PDIA. This result was further proof that PDI and PDIA are the same enzyme.

Table 6. Activity of PDIV under various conditions as indicated by the formation of unsaturated oligouronides or the release of free reducing sugars from sodium polypectate (NaPP).<sup>a</sup>

DEAE purified PDIV	<u>Enzyme Activity</u>	
	$\mu$ moles unsaturated product <sup>b</sup>	$\mu$ moles reducing product <sup>c</sup>
A	3.53	3.84
B	2.20	5.45
C	0.27	0.20
D	0.27	4.44

A = PDIV+NaPP (1.2% in 0.05 M Tris-HCl buffer) at pH 8.5 + 1.0 mM Ca<sup>++</sup>

B = PDIV+NaPP (1.2% in 0.05 M acetate buffer) at pH 6.0 + 1.0 mM Ca<sup>++</sup>

C = PDIV+NaPP (1.2% in 0.05 M Tris-HCl buffer) at pH 8.5 without ions

D = PDIV+NaPP (1.2% in 0.05 M acetate buffer) at pH 6.0 without ions

<sup>a</sup>Reaction mixtures were incubated for 16 hr at 30 C

<sup>b</sup>Expressed as the  $\mu$ moles of unsaturated oligouronides formed per ml of enzyme solution per hour

<sup>c</sup>Expressed as the  $\mu$ moles of free reducing sugars released from NaPP per ml of enzyme solution per hour

## DISCUSSION

Previous work in this laboratory has only been concerned with one pectic enzyme from Erwinia carotovora, an extracellular endopolygalacturonate trans-eliminase (39,61). This investigation, however, clearly demonstrated the presence of three intracellular polygalacturonate acid trans-eliminases as well as an oligogalacturonate acid trans-eliminase in Erwinia carotovora (Jones) Holland isolate 14. Also of major significance was the discovery that a single, highly purified pectic enzyme could exhibit dual enzymatic function. PDI and PDIIa (PGTE, pI 9.4 intracellular and extracellular, respectively) at pH 8.5 in the presence of calcium cleaved sodium polypectate (NaPP) in a trans-eliminative manner resulting in unsaturated products. PDI and PDIIa at pH 6.0 in the presence or absence of divalent cations cleaved NaPP in a hydrolytic manner resulting in saturated products. These enzymes had endo modes of depolymerization and from all the experiments conducted, including immunological comparisons, PDI and PDIIa appear to be the intracellular and extracellular forms of the same enzyme.

This isolate also produced two enzymes which exhibited exo modes of depolymerization. PDII (PGTE, pI 8.0) and PDIII (PGTE, pI 6.3) showed activity from pH 5.5-10, with optimal activity at pH 8.5. PDII required manganese for activity. Though PDIII activity was enhanced by the divalent cations  $Mn^{++}$ ,  $Ca^{++}$  and  $Mg^{++}$ , it did not require an ion as did PDI, PDIIa and PDII.

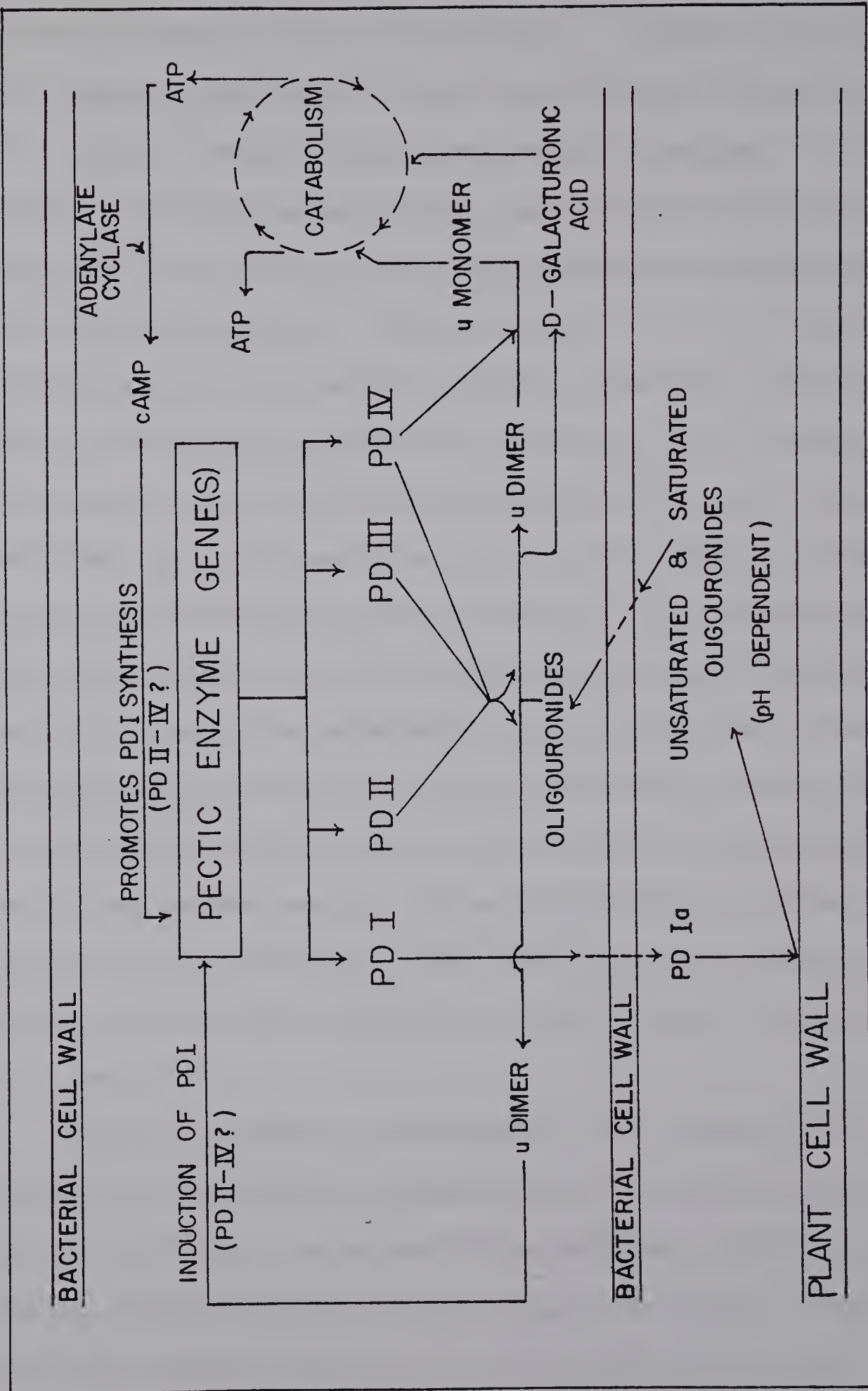
PDIV exhibited characteristics similar to the oligogalacturonate

acid trans-eliminase described by Moran et al (38). Though PDIV could depolymerize NaPP in an exo manner at a very slow rate, it easily converted unsaturated digalacturonic acid into presumably 4-deoxy-5-hexoseulose uronic acid (unsaturated monomer).

Moran et al (37) experienced difficulty in separating hydrolase and lyase activity. In light of our findings, a closer examination of Isolate 153 might be rewarding. It is possible that their second peak of lyase activity (from CM cellulose column chromatography) which was contaminated with hydrolase activity might be a single enzyme with dual function and should not have been discarded. It is also possible that Isolate 14 is unique and that other Erwinia carotovora isolates will not show the same characteristics. Erwinia chrysanthemi isolate 307 and isolate 23 were examined in a similar manner as isolate 14 and they showed no such capacity for production of an enzyme with dual function. However, the differences between E. chrysanthemi and E. carotovora are great. Isolates 307 and 23 induce a slow wilting in carnation whereas isolate 14 induces soft rotting of potato tubers. These are two very different types of disease. Isolates 307 and 23 both produce more than one extracellular trans-eliminase. Isolate 14 produces only one extracellular trans-eliminase. The number of trans-eliminases produced as well as the number secreted may be important to the type of disease a pathogen induces in its host.

The significance of these findings might best be demonstrated in the establishment of a model system upon which further work and evaluation can be based (Fig. 7). Erwinia carotovora has been shown to exist

Fig. 7. Proposed model for the pectic enzyme complex of Erwinia carotovora isolate 14 and its interaction with plant cell walls.



in symptomless potato tubers (Kelman, A., personal communication) showing no signs of a soft rot infection. A change in the micro-environment, whether due to insect wound, bruise, or senescence can transform the latent bacterium into an active pathogen. Isolate 14 secretes an endopolygalacturonate trans-eliminase (PDIa) which attacks the pectic acid fraction of the middle lamellae releasing many unsaturated oligouronides. PDIa can further act on the varying chain length oligouronides eventually yielding unsaturated digalacturonic acid. These smaller oligouronides (unsaturated dimer through perhaps pentamer) are then transported into the bacterium where they are acted upon by the intracellular pectic enzyme complex. The exo enzymes (PDII and PDIII) serve to convert all the oligouronides into unsaturated dimer. As a side product, trace amounts of galacturonic acid is released. Any galacturonic acid produced can be immediately assimilated into the energy yielding fermentation process (27). Under aerobic conditions pyruvic acid can pass into the tricarboxylic acid cycle yielding more energy. The unsaturated dimer produced by the exo enzymes is then converted by PDIV (OGTE) into 2 molecules of unsaturated monomer. This monomer can also be assimilated into the energy yielding food chain (38).

Some of the adenosine triphosphate (ATP) resulting from either fermentation or oxidative phosphorylation is converted by adenylate cyclase into cyclic adenosine-3',5'-monophosphate (cAMP) and pyrophosphate. The pyrophosphate can act to stimulate certain enzymes of the glycolytic pathway enhancing the continuation of the process. Cyclic AMP is necessary for synthesis of trans-eliminase (at least PDI and PDIa)

(22,40), therefore, the supply of cAMP allows continued synthesis of trans-eliminase. Inducer is also necessary. Although the absolute identity of the molecule which interacts at the DNA level has not been identified, the unsaturated dimer does lead to induction of trans-eliminase synthesis (24,62). As a result of the exo depolymerases, PDII and PDIII, there is unsaturated dimer available for the induction process.

Whether or not there is an operon containing the genes for some or all of the pectic enzymes of isolate 14 and whether or not these enzymes are under the same or a similar control mechanism remains to be established. All these enzymes are, however, produced when grown on pectic acid and all are approximately of the same molecular weight. Since they are present in the bacterium at the same time and they are all approximately the same size, there must be a positive recognition of PDI by a site on the membrane which selectively transports PDI out of the cell but not PDII, PDIII or PDIV. Recognition might be related to the enzymes antigenic properties or ionic charge.

From the point of view of survival, isolate 14 produces enzymes capable of activity in many environments. PDI and PDIA at high pH produce unsaturated oligouronides trans-eliminatively in the presence of calcium. At low pH they produce saturated oligouronides hydrolytically in the presence or absence of ions. PDII and PDIII produce unsaturated digalacturonic acid over a broad pH range and PDIII requires no ions. PDIV is capable of hydrolyzing the unsaturated digalacturonide into unsaturated monomers. This bacterium has the necessary enzymatic machinery to depolymerize the pectic acid fraction of plant cell walls,

utilize the breakdown products as a food source for energy, and generate the necessary molecules to maintain the synthesis and activity of these enzymes.

Although this organism produces multiple trans-eliminases, only one is of consequence to plant cell walls. PDIA, the probable extra-cellular form of PDI, is responsible for tissue maceration and cellular death. Controlling the soft rot disease caused by this organism might best be accomplished by controlling the synthesis and/or secretion of PDIA.

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