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THE EFFECTS OF *COLLETOTRICHUM LINDEMUTHIANUM* PECTIN LYASE ON BEAN TISSUE

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Abstract: A homogeneous preparation of pectin lyase (PL II, isoelectric point 9.7) from **Colletotrichum lindemuthianum**, caused the leakage of potassium ions from hypocotyl tissue discs of **Phaseolus vulgaris**. The enzyme preparation also released unsaturated uronic acids from pectin, isolated cell walls and hypocotyl tissue discs. Analysis of degradation products shows that PL II is an endo-acting enzyme.

Key words: Colletotrichum lindemuthianum, Phaseolus vulgaris, Pectin lyase

INTRODUCTION

Colletotrichum lindemuthianum (Sacc *et* Magn) Bri. *et* Cav. affects the above ground parts of its host *Phaseolus vulgaris*, the common bean. Invasion and growth of the fungus result in anthracnose disease in which large, water soaked necrotic lesions are produced.

C. lindemuthianum in culture produces pectic enzymes. These are, two forms of pectin lyase having isoelectric points of 8.2 (PL I) and 9.7 (PL II) and a single form of polygalacturonase having a pI of 9.3.[1,2] However, in *P.vulgaris* hypocotyl tissue infected with the fungus, PL II is the only pectic enzyme detected.[1,2] Pectic enzymes are known to be toxic to host cells and they play a major role in the development of necrotic symptoms in many diseases.[3] It is possible that the PL II of *C. lindemuthianum* also plays a similar role in bean anthracnose disease.[2] This paper describes the purification of PL II from *C. lindemuthianum* and the effect of the purified enzyme on host cells.

METHODS AND MATERIALS

Growth of the fungus: Race O of *C. lindemuthianum*[4] was grown in an ammonium tartrate medium[5] with sodium polypectate (Sigma, St. Louis, USA) as the main source of carbon. The medium was buffered with 0.1M NN' - Bis - (-2-hydroxyethyl)-2 amino ethane sulphonic acid (BDH, London) and the pH adjusted to 6.5 with 0.1M NaOH. Twenty five ml of the medium was dispensed into 200 ml medicine bottles and autoclaved. Inoculation and incubation was performed as described by

Abbreviations - PL : Pectin lyase, PG : Polygalacturonase, pI : Isoelectric point, TBA : Thiobarbituric acid.

Wijesundera *et al.*[1] Cultures were harvested 15 d after inoculation by filtration through Whatman no 1 filter paper. The culture filtrates were dialysed (using Spectrapor-2 membrane tubing) at 4°C for 24 h against distilled water and concentrated by freeze drying.

Ion-exchange chromatography: CM-Sephadex C -50 (Pharmacia, Sweden) was used for ion-exchange chromatography. The gel, equilibrated in 0.1M sodium acetate buffer, pH 4.5, was packed into a 16.0 x 2.0 cm^2 column according to the manufacturer's instructions.

The dry culture filtrate, re-dissolved in 15 ml of 0.1M sodium acetate buffer, pH 4.5, was applied to the gel and the gel was sequentially eluted with 50 ml of different 0.1M sodium acetate buffers of pH 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0 respectively. This was followed by a final wash with 50 ml of 0.1M sodium acetate, pH 7.0 containing 0.3M NaCl. Ten ml fractions were collected using gravity flow and assayed for enzyme activity. Protein content of the fractions were determined as described below.

Isoelectric focusing: Isoelectric focusing was carried out in an LKB 110 ml column using either Ampholine (LKB, Sweden) of pI range 3.5-10.5 or Pharmalyte (Pharmacia, Sweden) of pI range 7.5-10.5 as the carrier ampholytes in a linear sucrose gradient at 10^0C for 24 h. The column was eluted using a peristaltic pump at 120 ml h^{-1} and 3.0 ml fractions were collected. The pH of the fractions was measured immediately.

Isoelectric focusing was also carried out using pre-coated Servalyte precotes (Serva Finebiochemica GMBH, Heidelberg, Germany) as instructed by the manufacturers. The proteins were fixed by placing the precotes in a 3% solution of perchloric acid for 30 min to 1 h. To stain the fixed proteins the precotes were then placed in a solution of Coomassie blue G-250 (BDH, London) in 5% perchloric acid for 24 h. This was followed by the removal of excess stain by immersing the gel in a running water bath for 24 h. The washed precotes were dried at room temperature. The following proteins were used as markers: Cytochrome-c (horse heart) pI 10.8; Myoglobin (equine) pI 7.3 and carbonic anhydrase, pI 6.5 (Sigma, St.Louis, USA).

Growth of seedlings of *P. vulgaris* and preparation of cell walls: Seedlings of *P. vulgaris cv* Kievitsboon Koekoek were grown as described by Wijesundera *et al.*[1] Cell walls were prepared from hypocotyls of seedlings that were harvested 10 d after sowing.[1]

Determination of cellular permeability changes: Twenty 1.0 mm thick hypocotyl discs (approximately 0.05g/ disc) obtained from 7-day old *P. vulgaris* seedlings were used in each experiment. Before use, the hypocotyl discs were rinsed in the bathing solution

for 1 min. Leakage of K^+ from the hypocotyl discs was used to detect permeability alterations.

The bathing solution used to measure K^+ was 0.01M Tris-HCl buffer, pH 8.5. The rinsed discs were transferred to the test solution which included the enzyme sample in 8.0 ml Tris-HCl buffer. The K^+ concentration of the bathing solution was measured soon after the addition of the discs and at specified time intervals using a flame photometer (Model 1A, Evans Electro Selenium Ltd., U.K.). Other discs were treated with an enzyme sample inactivated by boiling for 10 min. All experiments were conducted at 20^0C. At the end of the study period the discs together with the bathing solutions were boiled for 5 min and after cooling the K^+ concentration was measured. This value was taken as the total K^+ concentration, and results are expressed as a percentage of the total K^+ concentration.

Purification of pectin: A method described by Archer[6] was used to purify pectin. Pectin (H.P.Bulmer, Hereford, UK) was washed several times with acidified (0.1M HCl) 80% v/v ethanol until the filtrate was free of pigments and reducing sugars. Nelson's[7] method was used to test for reducing sugars. After two more washings with ethanol the residue was collected by suction filltration, and dried using absolute ethanol:ether (1:1 v/v). Traces of solvent were removed by storing in a vacuum desiccator followed by heating to 60^0C. The resulting solid was ground to a fine powder and stored at 20^0C.

Action of PL II on different substrates: Action of purified PL II preparation on three different substrates - purified pectin (0.002 g), isolated hypocotyl cell walls of *P. vulgaris* (0.05 g), hypocotyl tissue discs of *P. vulgaris* (20 discs, each 1.0 mm thick) were tested. The tissue discs were from 7-day old seedlings and were washed for 1 h in 8.0 ml of the buffer used in the experiment. During washing the buffer was changed every 20 min.

Purified enzyme preparation (2.5 units) in 8.0 ml of 0.1M Tris-HCl buffer, pH 8.5 containing the antibiotic gentamycin sulphate (50 μg ml^{-1} Sigma, St. Louis, USA) and 0.01M $CaCl_2$ was used to treat each sample. Boiled inactivated enzyme was used as the control.

The reaction mixtures were incubated in a reciprocal shaker (100 strokes min^{-1}, each stroke 1.1 cm) at 25^0C. Samples were taken immediately after the addition of the enzyme and at intervals thereafter. The samples were placed immediately in a boiling water bath for 20 min to terminate enzyme activity and the cooled samples were examined for reducing sugars,[7] uronic acids,[8] and 4-5 unsaturated uronic acids by the thiobarbituric acid method[9] and by absorbance at 240 nm.[5] Activities are expressed as arbitrary units based on the absorbance after necessary corrections for substrate

blanks. The cooled samples were also subjected to both thin layer and paper chromatography as described below.

Enzyme assays: Pectin lyase (PL) activity was measured spectrophotometrically by the change in absorbance at 240 nm.[1] The standard reaction mixture comprized 3.0 ml 0.25% citrus pectin in 0.1M Tris-HCl buffer of appropriate pH and 0.1 ml of enzyme sample. The absorbance was read immediately afterwards and after an incubation period at 30°C. One unit of enzyme activity is defined as that amount which produce 1 μ mol unsaturated uronic acid in 1 min based on the molar absorption for the product of 4600. PL activity was also examined using the TBA method.[1] After specified reaction periods, 1.0 ml samples of the reaction mixture described above were added to 6.0 ml TBA reagent (5.0 ml 0.01M TBA and 0.1 ml 1M HCl) and kept in a boiling water bath for 60 min. After cooling the absorption was read at 550 nm.

Polygalacturonase activity was measured by the cup-plate method and the viscometric methods described in Wijesundera *et al.* [1] In the cup-plate method a sodium polypectate agar gel (Sigma, St.Louis, USA) was used. Activities are expressed relative to an aqueous solution (1 mg ml^{-1}) of Pectinol 10M which was defined as having 100 units of PG activity ml^{-1}.

Protease: Protease activity was determined using hide-powder azure blue as the substrate.[2] The reaction mixture had 10 g hide powder azure blue (Sigma, St.Louis, U.S.A.), 5.0 ml Tris-HCl buffer of pH 8.5 and 0.5 ml of the enzyme sample in 25 ml plastic bottles. Incubation was at 37°C. After incubation for a specified period the reaction was terminated by filtration through Whatman no 41 filter paper. Absorbance of the filtrate was measured immediately at 595 nm. Necessary corrections were made for substrate blanks.

Estimation of proteins: Protein estimation was based on a dye-binding method.[10] Bovine-serum albumin (Sigma, St. Louis, USA) was used as the reference standard. To 0.8 ml standards and approximately diluted sample, 0.2 ml of protein dye reagent (Bio-Rad, Germany) diluted as instructed by the manufacturers was added. The absorbance was measured at 595 nm after 30 min incubation at 25°C.

Thin layer and paper chromatography: Ascending thin layer chromatography on DF-Fertigplatten Cellulose-F plates (20 x 20 x 0.01 cm^3, Merck) developed in butanol: formic acid 2:3 (v/v) for 4-5 h. Descending paper chromatography on Whatman no.1 paper, developed in butanol: acetic acid : water 2:1:1 (v/v/v) for 12-16 h.

The chromatographs were developed at room temperature in tanks equilibrated with respective solvents. Reducing groups were revealed as pink brown spots by

spraying with aniline phthalate reagent (0.93 g aniline and 1.66 g phthalate reagent in 100 ml of 1-butanol saturated with water) followed by heating to 100^0C for 15-30 min. Unsaturated compounds appeared pink when sprayed with a saturated aqueous solution of thiobarbituric acid.[11]

In all thin layer and paper chromatography experiments polygalacturonic acid; galacturonic acid (Sigma, St.Louis, U.S.A.) and 4- 5 unsaturated digalacturonic acid was used as reference standards. The 4-5 unsaturated digalacturonic acid was prepared according to the method described by Nagel & Vaughn.[12]

RESULTS

Purification of PL II: When the fungus was grown in sodium polypectate medium buffered at pH 6.5, the only pectic enzymes detected were the two lyases (PL I and PL II). No PG activity was detected. The concentrate of the culture filtrate in sodium acetate buffer of pH 4.5, when subjected to ion-exchange chromatography gave the elution pattern shown in Figure 1. The PL resolved into two peaks, peak 1 corresponding to PL I and peak II to PL II. A peak of protease activity occurred just after the highest PL II activity, but the fractions having the highest PL activity were devoid of protease activity (Figure 1). The Fractions 25-28 were bulked, dialysed (using Spectrapor-2 membrane tubing) and concentrated by freeze drying. On servalyte precotes this PL preparation gave only one band when stained for proteins. The position of the protein band indicated a pI value corresponding to the pI of PL II.

The PL II prepared from ion-exchange chromatography on narrow range isoelectric focussing yielded a well defined peak of pI 9.7. This preparation is referred to as purified PL II. Details of the purification are given in Table 1.

Effect of PL II on permeability of cells: The purified PL II preparation caused a leakage of K^+ from tissue discs, and the amount and the rate of the ions leaked increased with the increase in activity of the PL II preparation (Figure 2). Statistical analysis of the results confirms this observation (Table 2).

Degradation of substrates: Purified pectin, isolated cell walls and hypocotyl tissue discs when treated with 2.5 units of purified PL II released unsaturated uronic acids and reducing sugars (Figures 3 a, b, and c). At the end of the incubation period the hypocotyl tissue discs treated with the enzyme had lost their turgidity. In the controls where boiled, inactive PL II was used the release of the above compounds was not detected and the loss of turgidity was not observed.

When the products of purified PL II action on pectin were subjected to thin layer and paper chromatography, the presence of a series unsaturated oligomers and very

Table 1: Purification of PL II of *C. lindemuthianum*

	Vol(ml)	PL activity (units ml^{-1})	Total PL activity	Protein μg ml^{-1}	Specific activity	Relative purification	% Recovery
Concentrated culture filtrate	15	372.0	5580.0	330.0	1.1	1.0	100.0
CM-Sephadex (Fractions 25-28)	40	74.4	2976.0	2.3	32.3	28.9	53.3
Isoelectric focusing (pI 7.5-10.5)	3	32.8	98.4	0.72	45.6	40.7	1.8

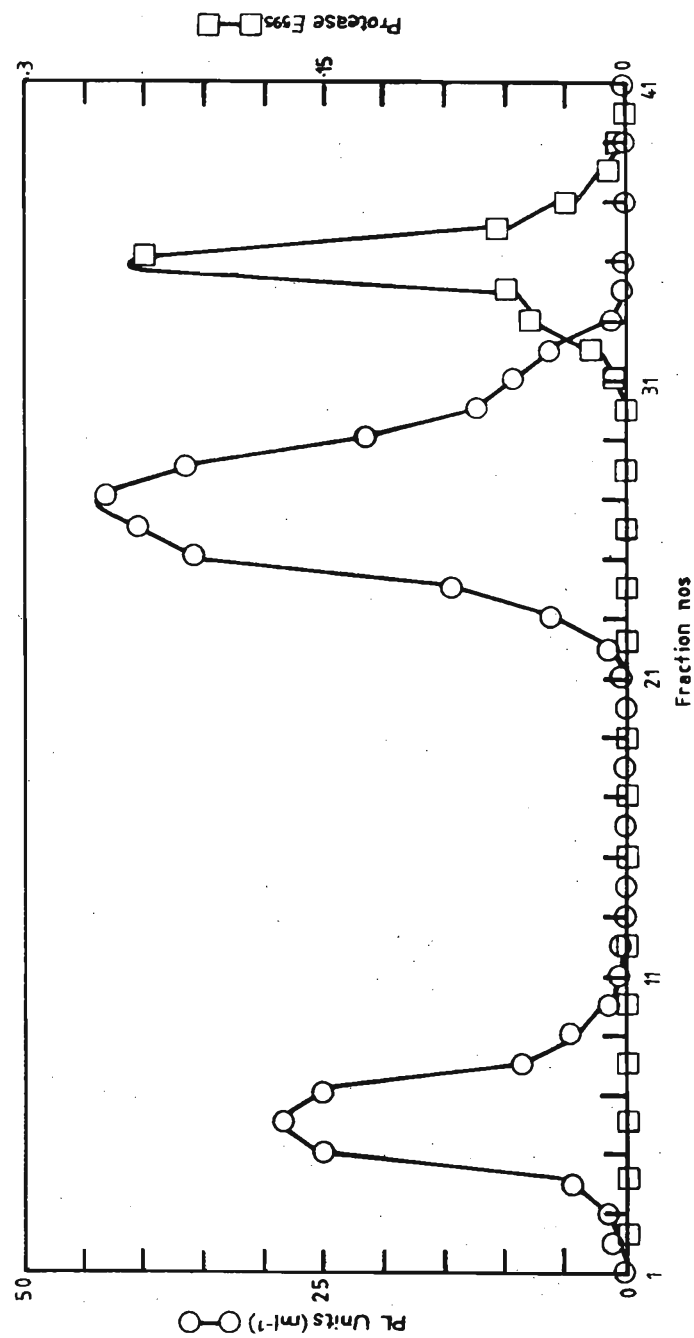


Figure 1: Elution pattern of the concentrated culture filtrate from sodium polypectate liquid medium buffered at pH 6.5, subjected to ion-exchange chromatography on CM-Sephades. PL-Pectin lyase.

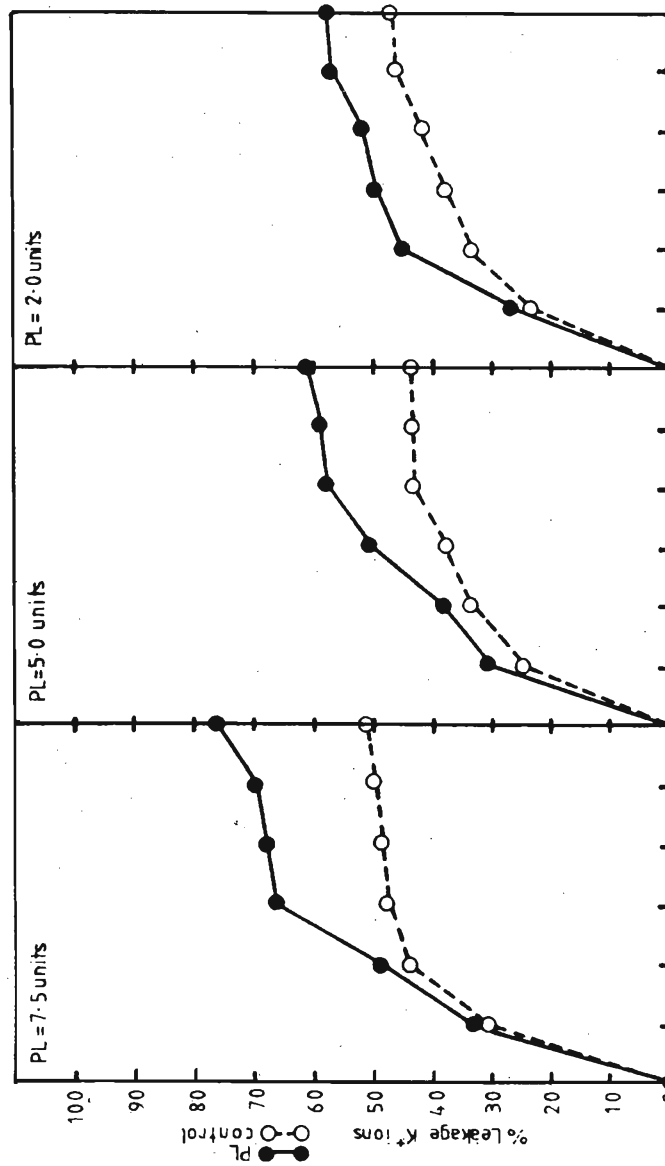
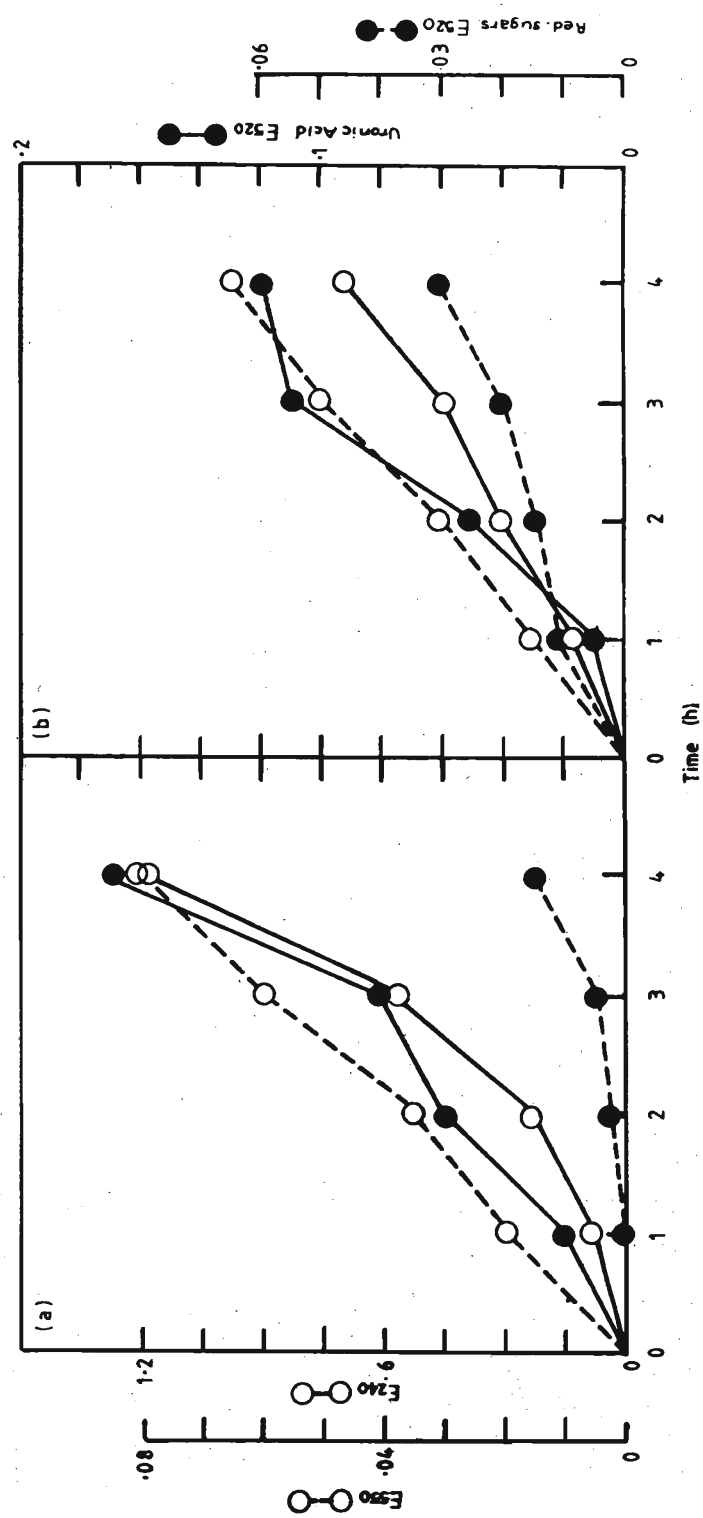


Figure 2: Leakage of K^+ from hypocotyl tissue in 0.01M tris-HCl buffer, pH 8.5 treated with purified PL II. Enzyme inactivated by boiling was used as the control.

a. Hypocotyldiscs treated with 7.5 units of PLII
b. Hypocotyl discs treated with 5.0 units of PLII
c. Hypocotyl discs treated with 2.0 units of PLII.


(a)
(b)
E540
E440
Uronic Acid E520
Red. sugars E520
Time (h)

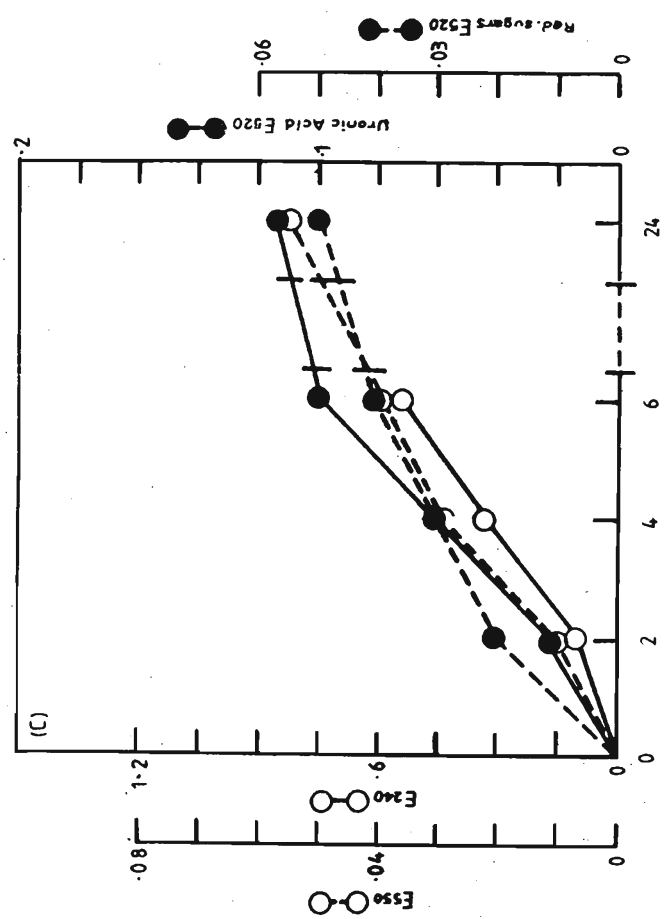


Figure 3: Products released by 2.5 units of purified PL II acting on,

a. 0.002g of purified pectin,
b. 0.05g of isolated hypocotyl cell wls,
c. Hypocotyl tissue discs (20 discs, each 1.0 mm thick) obtained from 7-day old seedlings, in 8.0 ml of o.1M tris-HCl buffer, pH 8.5
The reaction mixture also contained 50 μg ml^{-1} gentamycin sulphate and 0.01M $CaCl_2$.

E_{550} - TBA method for determination 0f 4,5 - unsaturated uronic acids.
E_{240} - Determination of 4,5 - unsaturated uronic acids by change in absorbance at 240 nm.
E_{520} - Nelson's method for determining reducing sugars.
E_{520} - Blumenkrantz and Asboe-Hansen method of determining uronic acids.

low levels of 4-5 unsaturated galacturonic acid and galacturonic acid were observed. With isolated cell walls and hypocotyl tissue discs the pattern was similar (Figure 4). The release of these compounds were not observed when boiled inactive enzyme was used.

DISCUSSION

The purification scheme proposed for PL II yielded a homogeneous preparation as determined by isoelectric focusing on Servalyte precotes. The purified PL II preparation caused leakage of K^+ from the cells of hypocotyls. The rate and amount of leakage increased with the increase in enzyme activity, thus confirming that the effect is due to enzyme action.

Table 2: Effect of PL II on ion leakage from tissue.

Treatment	% Leakage* (at end of expt)	Standard Error
PL = 2.5 units	58.4	1.06
Control	41.9	0.78
PL = 5.0 units	63.4	1.92
Control	43.2	0.80
PL = 7.5 units	78.1	2.41
Control	50.2	0.56

* Average of 3 replicates

For each level of PL, the value for treated tissue is significantly higher than the respective control at P = 0.05, by Student's t test.

The purified PL II degraded pectin, isolated cell walls and hypocotyl tissue discs generating 4-5 unsaturated uronic acids and reducing sugars. The loss of the turgidity of tissue observed also suggest tissue degradation. Analysis of degradation products by thin layer and paper chromatography showed the presence of large amounts of unsaturated oligomers among the reaction products. This suggests that the PL II is endo-acting.

It has been reported that the large scale death of cells and the collapse of *P. vulgaris* tissue during the transition from biotrophy to necrotrophy is associated with an increase in PL II activity and an increase in the pH of hypocotyl tissue.[2] The present results suggest the endo acting PL II brings about cell death in bean hypocotyl cells - by degrading cell walls and causing electrolyte leakage.

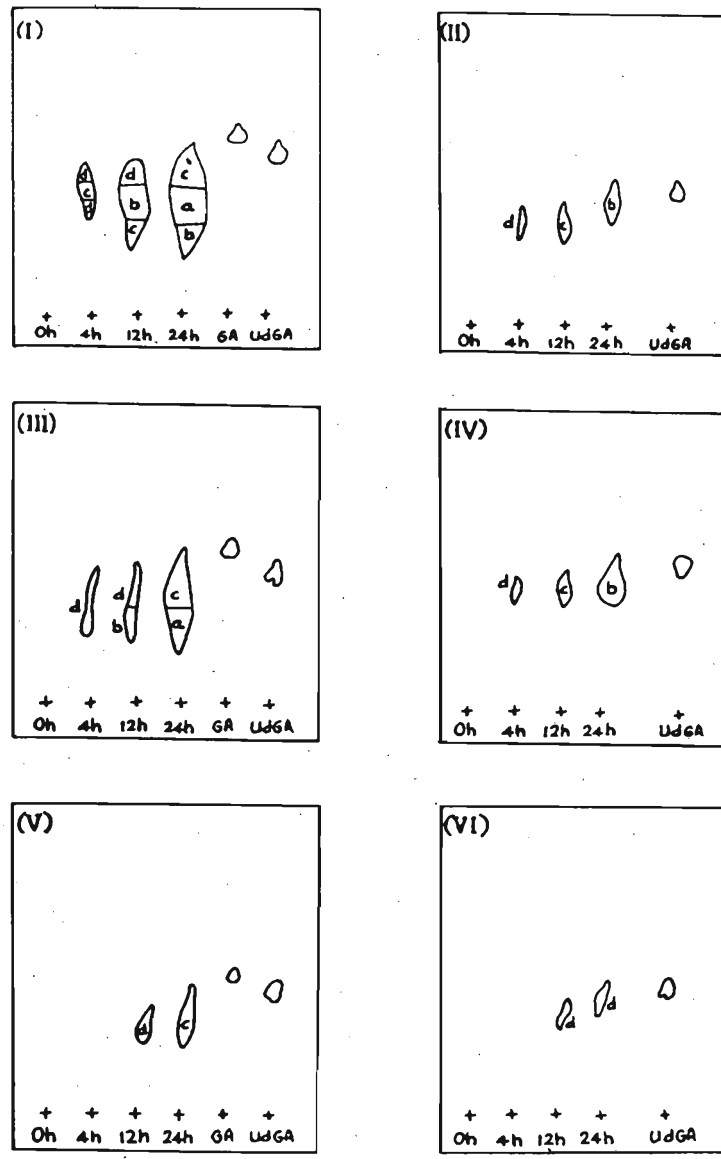


Figure 4: **Thin layer chromatographs of reaction products from purified PL action on, i and ii washed pectin, iii and iv isolated cell walls, v and vi tissue discs.**

i, iii and iv - plates sprayed with aniline phthalate to expose reducing sugars.
ii,iv and vi - plates sprayed with TBA to expose unsaturated uronic acids.

Intensity of spots, a - very high, b - high, c - moderate, d - low.

GA - galacturonic acid
UdGA - unsaturated digalacturonic acid
h - hours after start of reaction.

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