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CHARACTERIZATION AND PURIFICATION OF EXTRACELLULAR POLYGALACTURONASE COMPLEX PRODUCED BY *PHYTOPHTHORA PARASITICA* DAST.[1,2]

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Abstract

Phytophthora parasitica Dast. produced three polygalacturonase isoenzymes in culture filtrate, designated as endo-PG_1, endo-PG_2 and exo-PG with isoelectric points at pH 9.0, 7.3 and 3.5, respectively. Endo-PG_2 and exo-PG were further pruified by ammonium sulfate fractionation, gel filtration on Sephadex G-100 and isoelectric focusing, and enriched 242.9-fold and 289.7-fold, respectively. Both enzymes have a optimum pH at 5.0. Ca^{2+} concentration at 10^{-4} M partially inhibited the enzyme activities. Endo-PG_2 significantly caused tissue maceration and cell separation on cucumber discs as well as egg plant and potato tissues but exo-PG enzyme did not. However, when cucumber discs were treated with the combined enzyme preparation (endo-PG_2 + exo-PG) under the same condition, apparently the exo-PG did enhance the rate of tissue maceration. The fact that endo-PG_2 could cause cell separation as a result of releasing single cells from cucumber discs has been observed under light microscope as well.

Introduction

Phytophthora parasitica Dast. is one of the most common pathogens of considerable number of tropical and subtropical plants. This fungus causes different rotting types of seedlings, fruits as well as stems in field crops. Because it causes a soft rot at the infection site of its host tissue, the production of pectic enzyme by the fungus was expected, since this group of enzyme has been considered as a primary factor which causes a soft rot disease syndrome.

Studies on pectic enzyme of *Phytophthora* spp. have been reported in several instance. Cole (1970) demonstrated that filtrate from culture of *P.*

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infestans and inoculated potato tuber contained an endo-polygalacturonase (PG). The nutritional requirement of *P. palmivora* in relation to extracellular pectic enzyme were carefully studied by Akinrefon (1969). He found that both mycelial growth and enzyme production could be related to the incubation period, temperature, and the pH of the madia. The mycelial growth could not be related to the quality of enzymes produced, for instance, he indicated two peaks of PG and 2-L-arabinofuranosidase (AF) activities were recorded corresponding to the phases of optimum growth and autolysis of the mycelium, respectively. Recently, an endo-polygalacturonate trans-eliminase produced by *P. capsici* has been reported by Tseng and Tseng (1980), the purified enzyme caused tissue maceration and cellular death of egg plant and potato discs, but failed to induce permeability changes in both plant tissues.

There is little information concerning pectic enzyme which produced by *P. parasitica*, except Mclntyre and Hankin (1978) revealed that 10 of 14 isolates of the fungus produced polygalacturonase on solid media, but no pectate trans-eliminase was detected. This report deals with characterization and purification of multiple polygalacturonases produced by *P. parasitica* in cultural medium and their involement in the rot of plant tissues are also discussed.

Material and Methods

The isolate of *P. parasitica* Dast. originally isolated from egg plant fruit in field was kindly supplied by Dr. H.S. Chang. The cultures were either maintained in sterile distilled water for a long time presevation or on potato sucrose agar slant (200 g of potato extract, 40 g of sucrose 40 g and 12 g of agar, make up to 1 l, pH5.0; PSA) and transfers were made at 2-week intervals.

Enzyme source

In order to obtain uniform inocula of the fungus for producting pectic enzyme in cultural medium, subcultures of the isolate on PSA plates which were incubated at 28°C for 5-day were used. Standard inoculum discs were then cut with a cork border (0.4 cm Dia.) from the advancing edge of the colony in the plate. The cultural medium served for the enzyme preparation consisted of 5.0 g of peptone, 2 g of KH_2PO_4, 25 g of $MgSO_4 \cdot 7H_2O$, 15 mg of thiamine-HCl, 15 g of sucrose and 250 ml of cucumber fruit extract in 1 l of distilled water. The initial pH was adjusted to 5.0, and the medium was then dispensed in 100-ml Erlenmeyer flask. These flasks were autoclaved for 15 min at 15 lb/in^2 pressure. Five discs of standard inoculum were then transferred to each flask and incubated with shaking at 28°C for a certain period of time. The culture filtrates were obtained by filtering the culture through Whatman

No. 1 filter paper and immediately assayed for pectic enzyme activity, and they were served as crude enzyme preparations for further investigation.

Enzyme Assay

Endo-polygalacturonase (endo-PG) was measured by cup-plate method as described by Dingle *et al.* (1963), using 1.2% each of pectin N.F. (Calbiochem product, Los Angeles 63, U.S.A.) and sodium polypectate (Nutritional Biochemical Cooperation, Cleveland, Ohio, U.S.A.; Napp) as substrates. The specific activity (S.A.) of the enzyme was expressed as follow equation:

$$\text{S.A.} = \frac{2^{t-1}}{100 \times \text{protein content}}$$

t = D−9 mm(9 mm is diameter of cork border)

where D is clear zone diameter (mm) in agar plate after 24 hr incubation at 30°C.

The enzyme activity was also assessed by viscometric method, since endo-type of pectic enzyme used to cleavage its polymer substates in random way, resulting in a rapid loss of the substrates in reaction mixtures. Fenske-Ostward viscometer was served for this purpose. Reaction mixture contained 4.0 ml of 0.6% Napp or pectin buffered with 0.05 M of various buffers at different pH values (citrate-phosphate buffer, pH 3–5; phosphate buffer, pH 6–7 and Tris-HCl buffer, pH 8–11) plus 2 ml of distilled water and 1 ml enzyme preparation (Bateman, 1966). The measurements were carried out at 30°C. The enzyme activity was expressed in relative activity units ($1/t \times 10^3$, where t is the time in minutes for 50% viscosity loss).

Exo-polygalacturonase (exo-PG) activity was determined by measuring the rate of increase of reducing sugars in reaction mixture (Nelson, 1944). Reaction mixtures contained 0.1 ml of 0.6% Napp or pectin in 0.05 M citrate-phosphate buffer (pH 5.0) and 0.1 ml enzyme preparation at 30°C for 1 hr. One unit of the enzyme activity is defined as that amount of enzyme increase in the release of 10 μg galacturonic acid in 60 min at 500 nm in Nelson test.

Polygalacturonate trans-eliminase (PGTE) and pectin methyl trans-eliminase (PMTE) activities were measured by the periodate-thiobarbituric acid (TBA) method (Presis and Ashwell, 1963), Reaction mixtures contained 0.1 ml of 0.6% Napp or pectin in 0.05 M Tris-HCl buffer (pH 8.0) plus 10^{-3} $CaCl_2$ and 0.1 ml enzyme preperation at 30°C for 1 hr. One unit of PGTE or PMTE activity was expressed as that amount of enzyme giving an increase in absorbance of 0.1 in 60 min at 548 nm in TBA assay. Autoclaved enzymes were served as controls, and protein content was measured by Lowry method (1951).

Enzyme Purification

Cultural filtrate (200 ml) was brought to 20% saturation with ammonium sulfate, allowed to stand for 1 hr at 4°C, and centrifuged for 20 min at 20,000 g. The precipetate was collected and dissolved in 10 ml of distilled water. This procedure was repeated for ammonium sulfate saturation to 40, 60, 80 and 95%. The precipetate of each fraction was assayed for pectic enzyme activity.

In the prelimenary study, since a bulk of polygalacturonases located in the 60–80% $(NH_4)_2SO_4$ fraction, thus, this fraction was applied to a 1.6×56 cm column of Sephadex G-100 buffered with 0.05 M citrate-phosphate at pH 5.0. The column was eluted with the same buffer at 4°C. Five-ml fractions were collected and aliquot of the fractions was assayed for the enzyme activity. The fractions which contained polygalacturonases were pooled and finally subjected to isoelectric focusing in a LKB 8101 Ampholine electrofocusing apparatus equiped with a 110-ml column using a pH 3.5–10 Ampholine as carrier (Tseng and Tseng, 1980). The purified enzymes were further analyzed by disc gel electrophoresis (Hedrick and Smith, 1968).

Estimation of tissue maceration by polygalacturonase of P. parasitica

Tissue maceration was evaluated and rated as described by Mount *et al.* (1970). Cylinders of fully turgid potato tuber, cucumber and egg plant fruit tissues were cut with a cork-borer (9 mm dia), and discs (1 mm thick) were cut with a hand microtome and washed in distilled water. The washed discs were placed in reaction mixture (6 discs/ml) containing equal volume of buffer and enzyme at 28°C. Measurements of tissue maceration were made over 9-hr incubation period. Discs which were not macerated received a rating of 0, which were completely macerated received a score of 5.

Result

Cultural filtrates of *P. parasiica* which grew in the medium with cucumber extract as enzyme inducer were tested for the production of extracellular pectic enzyme by the fungus. Since pH value of cultural medium and the incubation period of the culture may influence the growth of fungus as well as its ability to produce pectic enzyme, thus, in the prelimenary study, effects of pH and optimal growth condition of the fungus to induction of pectic enzyme were investigated.

Changes in pH values of the cultural medium inoculated with *P. parasitica* and its dry weight of mycelial mass have been found during 14-day incubation at 28°C. The data presented in Fig. 1. showed that the initial pH of the cultural medium was 5.6 and then became more acidic at pH 4.5–5.0. The optimum pH for the fungus growth during incubation period was around pH

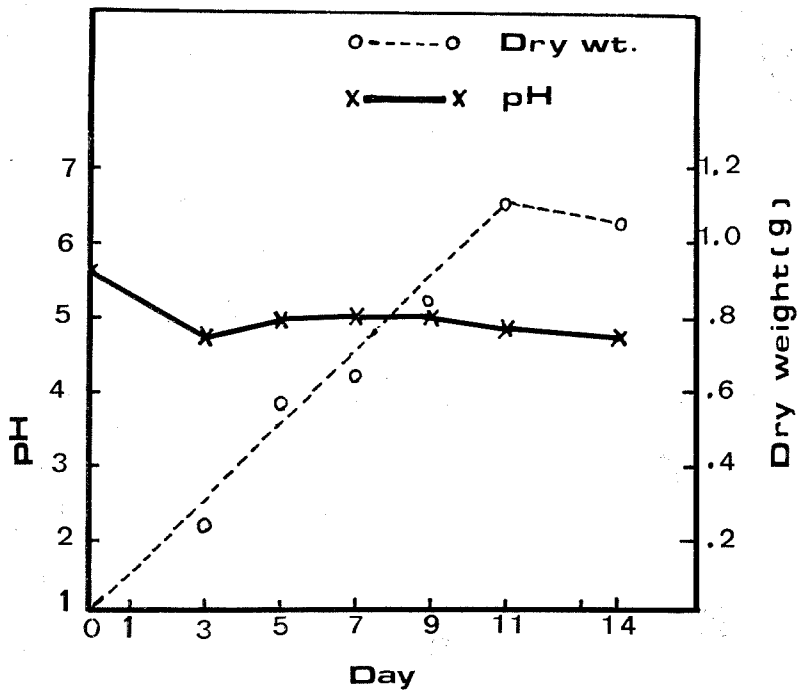

Fig. 1. Changes in pH value and dry weight of *Phytophthora parasitica* in the culture medium with cucumber extract.

5.0, this is also an adequate pH for pectic enzyme activity. Dry weight of the mycelial mass in the culture medium, however, was drastically increased as the incubation time elapsed, until it reached to 11-day and then decline.

Using viscometric assay to determing the ability of *P. parasitica* to produce pectic enzyme during a 14-day incubation was illustrated in Fig. 2. It was apparently elucidated that the growth of fungus in the culture medium started earlier than the enzyme production (Fig. 1 and 2). There was a sharp rising of the enzyme production during the period corresponding to the logarithmic growth phase, and the pectic enzymes being detected in the culture medium obviously prefered the substrates of Napp and pectin at pH 5.0 rather than at pH 8.0. It indicated that the pectic enzymes existed in the culture medium could be classified as pectic glycosidase group. This has been further confirmed by using TBA assay to search for any lyases in culture filtrate but failed.

pH optima of the pectic glycosidases produced by *P. parasitica* in culture medium have been extensively studied. Results (Fig. 3) illustrated that the enzymes exhibited a high activity at acidic range (pH 3.0–6.0) and it had a substrate preferring to Napp than pectin, except at pH 3.0. This indicated that two different types of pectic glycosidase such as polygalacturonase (PG) and pectin methyl galacturonase (PMG) may be existed. In contrast, no

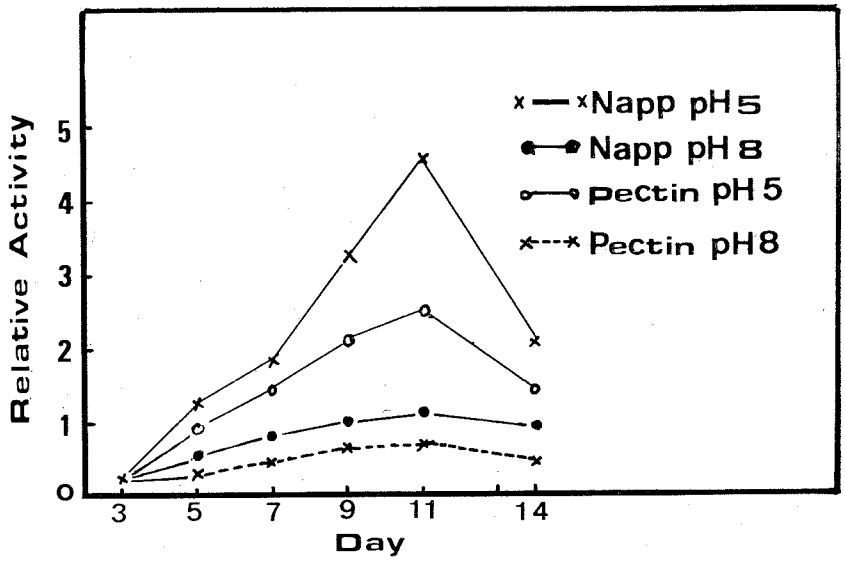

Fig. 2. Pectic enzymes of *Phytophthora parasitica* in culture medium at various incubation periods. The enzyme activity was measured by viscometric assay.

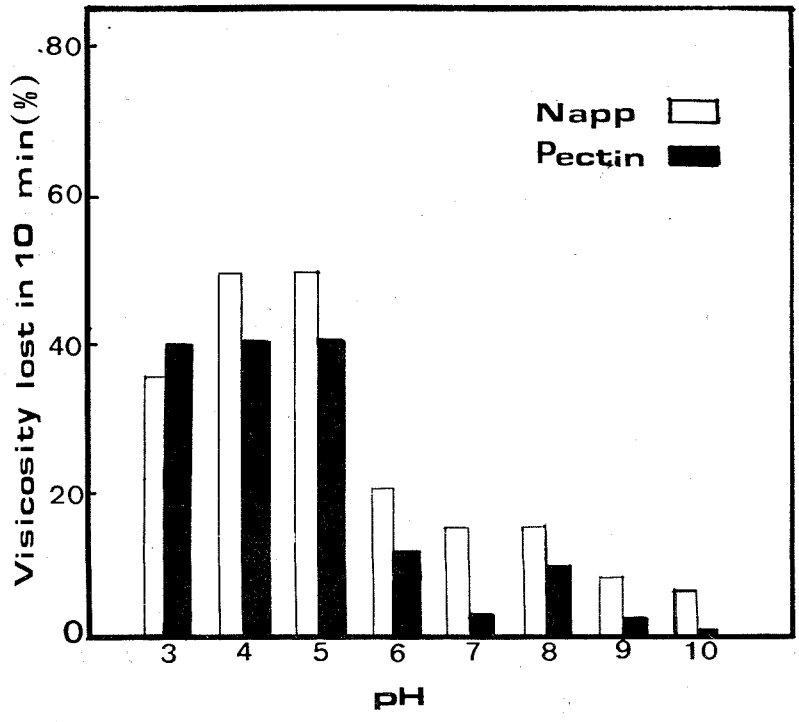

Fig. 3. pH optima of pectic enzymes produced by *Phytophthora parasitica*. The enzyme activity was estimated by viscometric assay.

appreciable activity of the enzyme was found when the pH reached to alkaline range. No lyases were detected by TBA assay in the culture filtrate cover the whole range of pH tested.

Purification of *P. parasitica* pectic glycosidases was carried out as the following manner. Culture filtrate (250 ml) of the fungus which contained a considerable amount of pectic glycosidases was subjected to ammonium sulfate fractionation. Precipitates in 0–20%, 20–40%, 40–60%, 60–80% and 80–95% saturated solutions were collected by centrifuging at 20,000 g, dissolved in 10 ml of cold distilled water and dialysed against distilled water at 4°C for 24 hr.

The precipitate from the 60–80% fraction contained most of the pectic glycosidases was assayed by cup-plate and viscometric methods. Five ml of the dialysed fraction was then fractionated on a column of Sephadex G-100 superfine eluting with 0.05 M citrate-phosphate buffer (pH 5.0). Results (Fig. 4) showed the pectic glycosidases were mainly located within the fractions of 10–15, the enzyme activities were measured by Nelson and cup-plate methods. Suprisingly, only Na-polypectate (pH 5.0) used as substrate showed the enzyme activity, however, no appreciable enzyme activity was found when pectin (pH 5.0) was served as substrate. These results illustrated the pectic

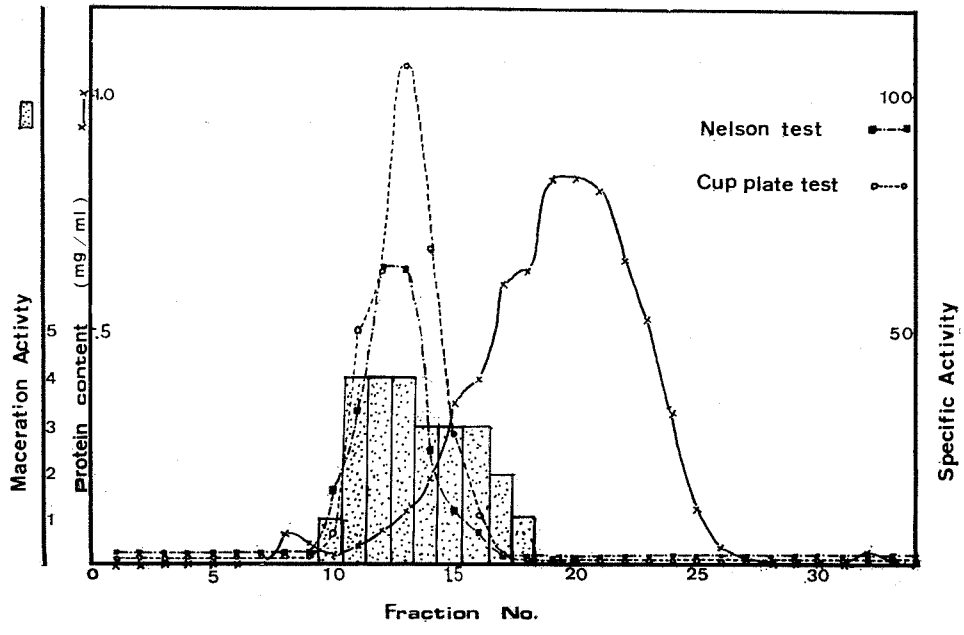

Fig. 4. Gel filtration pattern of *Phytophthora parasitica* polygalacturonase in the 60–80% ammonium sulfate fraction of culture filtrate on Sephadex G-100 column. All of the fractions were checked for its maceration activity by using cucumber discs. Five-ml fractions were collected, and polygalacturonase was measured by Nelson test and cup plate method.

glycosidases in culture filtrate of *P. parasitica* was PG but not PMG. The reason for the missing of PMG after passing through Sephadex G-100 Column, which might exist in the culture filtrate, will be discussed later. The fractions from the Sephadex column were checked for its ability to cause tissue maceration, only fractions from 10–18 exhibited positive results, and it correlated very well with the fractions which contained polygalacturonases.

Fractions which contained most of the polygalacturonases, No. 10–15, were finally subjected to isoelectric focusing by using an Ampholine carrier with a pH range of 3.5–10. Three sharply defined PG fractions were obtained and it was well separated (Fig. 5). The PI values of three polygalacturonases were 9.0, 7.3 and 3.0, respectively. The purified enzymes were further characterized by Nelson test in order to measure reducing sugars which are rapidly released by exo-polygalacturonase in reaction mixture, and by viscometric assay which is usually used to detect endo-polygalacturonase type of enzyme based on its causing a rapid loss of viscosity in its polymer substrate such as

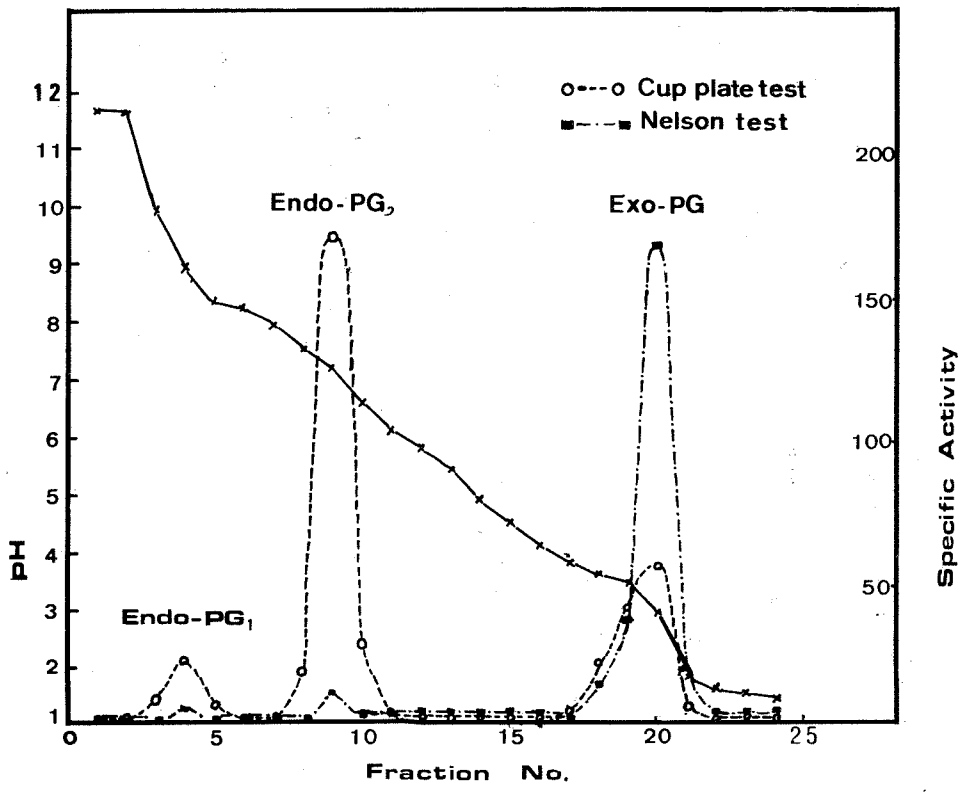

Fig. 5. Separation of endo-and exo-polygalacturonases from Sephadex G-100 column (Fig. 4, fraction No. 10–15) by electrofocusing with a LKB 110 ml column using Ampholine carries with a pH rang of 3.5–10; 5 ml-fractions were collected. The enzyme activities were measured by Nelson test as well as cup-plate method.

Na-polypectate. In addition, cup-plate method was also served for measuring both enzyme activities. Since peak 1 and 2 caused a rapid loss of viscosity in its substrate (Napp at pH 5.0) in 10 min as compared with Peak III and released less amount of reducing sugars in reation mixture as showed in Fig. 5, Therefore, both peak 1 and 2 enzymes were confirmed as endo-type of polygalacturonase and designated as endo-PG_1 and endo-PG_2. On the contrary, peak III enzyme was characterized as exo-PG.

Only the purified endo-PG_2 and exo-PG of *P. parasitica* were finally subjected to disc-gel electrophoresis for evaluating its homogeneity. Results (Fig. 6) showed that two enzymes were not well separated presumablely they have a similar molecular weight. However, which enzyme, endo-PG_2 or exo-PG, corresponding to a or b band in the gel was not characterized in this study. Both purified polygalacturonases have a pH optium at the range of 4–5. The enzyme activities were partially inhibited by Ca^{2+} at 10^{-4}M concentration however, it was completely inhibited when Ca^{2+} ion reached to 10^{-3} M for endo-PG_2 and 10^{-2} M for exo-PG (Fig. 7).

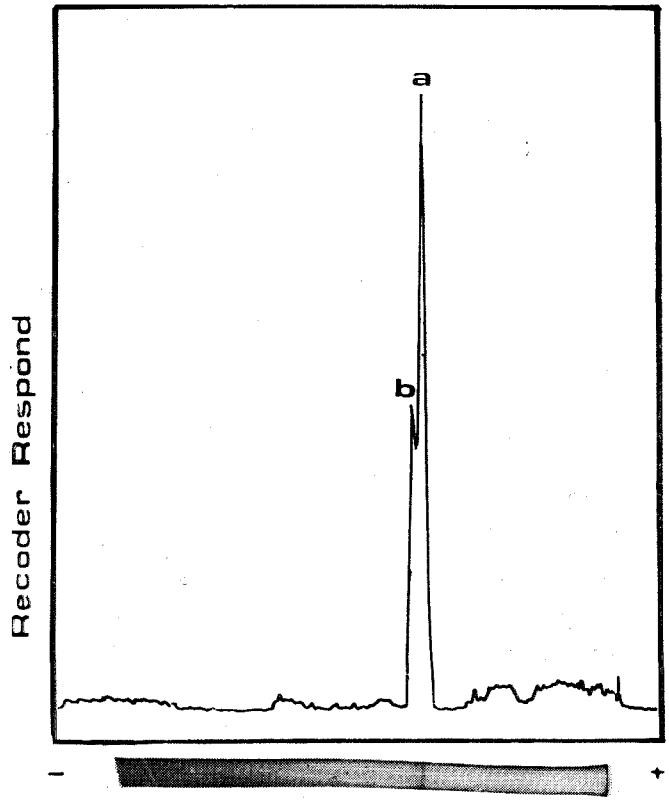

Fig. 6. Separation of endo-PG_2 and exo-PG by disc-gel ele ctrophoresis (the purified PG from Fig. 5). Electrophoretic gel was scanned by Gilford 2400-S Spectrophotometer at 550 nm.

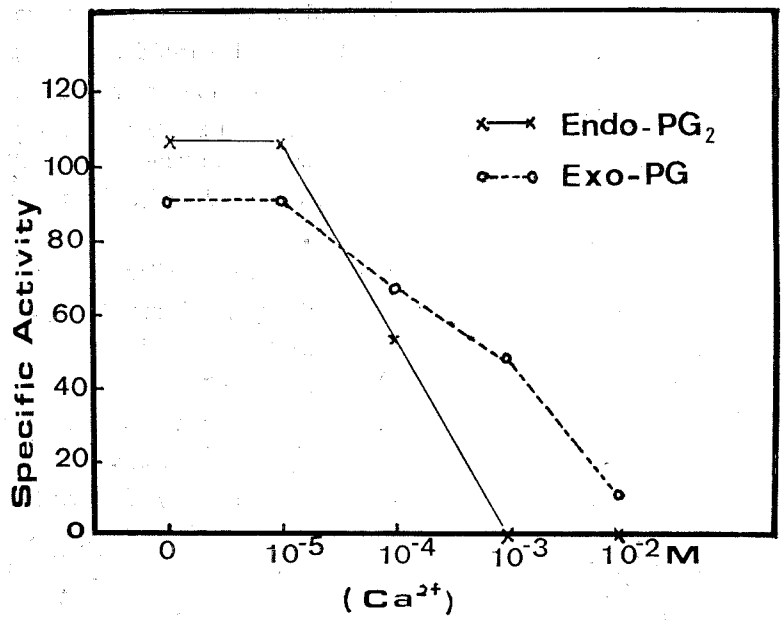

Fig. 7. Effect of calcium on the activities of Endo-PG_2 and Exo-PG of *Phytophthora parasitica*. Endo-PG_2 was assayed by cup-plate method and Exo-PG was measured by Nelson test.

Endo-PG_2 in the culture medium was finally purified to 242.9-fold and 289.8-fold for exo-PG by 60–80% $(NH_4)_2SO_4$ fractionation, Sephadex G-100 column chromatography and isoelectric focusing.

Effect of *P. parasitica* pectic enzymes on plant tissues was carried out in the current study. The responses of loss in coherence of discs from different cultivars such as egg plant, cucumber and potato treated with the enzyme preparations of crude and ammonium sulfate fraction of the fungal culture filtrate were not identical (Table 1). Generally, crude and various $(NH_4)_2SO_4$ fractions of the pectic enzymes caused cucumber tissue maceration at pH 5.0 but not pH 8.0, except the enzyme preparation from 60–80% $(NH_4)_2SO_4$ fraction caused tissue maceration on all of the tested discs at pH 5.0 and pH 8.0. In addition, the crude enzyme preparation also caused potato tissue maceration at pH 5.0. Autoclaved enzyme preparation, however, did not caused tissue maceration. Table 2 summarized the results of purified *P. parasitica* polygalacturonases which induced tissue maceration on cucumber discs. Only endo-PG_2 significantly caused tissue maceration and cell separation on cucumber discs but exo-PG enzyme did not. However, when cucumber tissue was treated with the combined enzyme preparation under the same condition, apparently the exo-PG did enhance the rate of tissue maceration. The purified endo-PG_2 which caused cell separation as a result of releasing single cells from cucumber discs has been observed under light microscope (Fig. 8).

Table 1. *Effects of crude and various ammonium sulfate fractions of* ***Phytophthora parasitica*** *pectic enzymes on plant tissue maceration*

Enzyme	Crude			% $(NH_4)_2SO_4$ Fraction												Auto-claved		
	0-20			20-40			40-60			60-80			80-95					
Plant tissue (hr)	a	b	c	a[1]	b	c	a	b	c	a	b	c	a	b	c	a	b	c
pH 5																		
1	0[2]	0	0	0	0	0	0	0	0	0	1	0	1	2	0	0	1	0	0	0	0
3	0	1	0	0	1	0	0	1	0	0	2	0	1	3	1	0	2	0	0	0	0
5	0	2	1	0	1	0	0	2	0	0	3	0	2	5	2	0	3	0	0	0	0
9	0	3	2	0	2	0	0	3	0	0	5	0	4	5	4	0	5	0	0	0	0
pH 8																		
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0	1	2	2	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0	0	0	3	4	4	0	0	0	0	0	0

[1] a→egg plant; b→cucumber; c→potato

[2] 0→no maceration; 5→completed maceration

Table 2. *Effect of purified endo-and exo-polygalacturonases of* ***Phytophthora parasitica*** *on cucumber tissue maceration at pH 5.0*

Time (hr) / Enzyme	2	3	4	5	6	8
Exo-PG	0[a]	0	0	0	0	0
Endo-PG	1	1	2	2	3	4
Endo-PG + Exo-PG	1	2	3	4	5	5

[a] The scale of maceration rate is the same as described in Table 1.

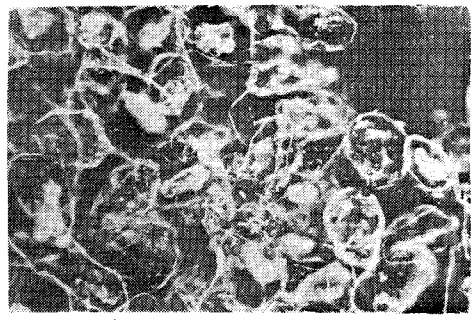

Fig. 8. Single cells released from cucumber discs after treating with purified *Phytophthora parasitica* endo-PG_2 at 28°C for 4 hr. (×350)

Discussion

Recently, the use of the isolectric focucing technique has made the purification and concentration of enzymes easier and enhanced the resolution of various isozymes. For instance, it is now evident that many phytopathogens produced multiple isoenzymes of polygalacturonase (Bateman 1972; Cervone *et al.* 1977; Fielding and Byrde 1969; Prssey and Avants 1973; Arinze and Smith 1979). However, research on pectic enzymes of *P. parasitica* Dast. on this line was still meager. In this study, we reported that *P. parasitica* produced three isoenzymes of polygalacturonase in culture filtrate. They are designated as endo-PG_1, endo-PG_2 and exo-PG, with isoelectric points at pH 9.0, 7.3 and 3.5, respectively. The purified endo-PG was able to cause tissue maceration and cell separation of cucumber discs at pH 5.0, but no significant tissue degradation was found when it was treated with purified exo-PG under the same conditon. (Table 2.)

In table I, we found the enzyme preparation from 60–80% $(NH_4)_2SO_4$ fraction of the culture fitrate caused tissue maceration on the discs of patato, cucumber and egg plant at pH 5.0 as well as pH 8.0. This is an ambiguous results because the optimum pH of *P. parasitica* pectic enzyme is around pH 4–5, almost no pectic enzyme activity was demonstrated at the alkaline pH range, theoretically, no tissue maceration will be found when the tissures were treated with the enzyme preparation of 60–80% $(NH_4)_2SO_4$ fraction at pH 8.0. It is suggested that the enzyme preparation may exist macerating factor(s) other than pectic enzyme which will induce tissue maceration at high pH value or the results are due to artifact. These questions remain for further investigation.

In Fig. 3. It was noted that the pectic enzymes of *P. parasitica* in culture filtrate exhibited a high enzyme activity at acidic pH range, and they prefer to the substrates of Napp and pectin. Therefore, it was suspected previously that there may have two types of pectic glycosidase e.g. PMG and PG presented in the enzyme preparation. However, when the culture filtrate was purified through ammonium sulfate fractionation and gel filtration, we failed to obtain any PMG in the fractions obtained from Sephadex G-100 column. this may explain why there was no any PMG involed in the culture filtrate of *P. paractica* but only polymethylesterase (PME), since PME is the enzyme which is able to cleavage the methyl group of pectin molecule as the result of releasing polygalacturonic acid which is the substrate of polygalacturonase. The point is that if any PME existed in the culture filtrate. This problem remains to be solved.

In the current study, we have also searched for pectic enzyme from diseased tissues of *P. parasitica* infected cucumber and egg plant fruits, surpri-

singly, only pectin methyl trans-eliminase (PMTE) and polygalacturonate trans-eliminase (PGTE) are extracted from both diseased tissues. Characterization and purification of the enzymes as well as its relation to rotting of its host plant tissue are in progress.

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對 *Phytophthora parasitica* Dast. 所產生之細胞體外聚合半乳醣分解酵素複合體之純化及其特性之研究

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Phytophthora parasitica Dast. 在培養液中，產生三種聚合半乳醣分解酵素之同功酶，分別命名爲 endo-PG_1, endo-PG_2 和 exo-PG，其等電點爲 pH 9.0, 7.3 和 3.5。經過硫酸胺鹽析法，膠質過濾色相分析法與等電點集中法，已將 endo-PG_2 純化爲 242.9 倍，exo-PG 純化爲289.7倍，兩者最適合之酸鹼度爲 pH 5.0，而且當鈣離子濃度爲 10^{-4}M 時，部份酵素活性受抑制；endo-PG_2 可導致黃瓜，茄子及馬鈴薯組織薄片之軟化及細胞之游離，而 exo-PG 則無此現象；然而當黃瓜之組織薄片用混合之 endo-PG_2 及 exo-PG 之酵素溶液在同一條件下處理時，發現 exo-PG 能促進組織軟化之速率；我們亦曾經在光學顯微鏡下觀察到因 endo-PG_2 引起黃瓜組織細胞游離，而自組織釋放出來的單一細胞。