한국식물병리학회지 10(3) : 163-168(1994)
Korean J. Plant Pathol. 10(3) : 163-168(1994)

Characterization of Pectate Lyase Produced by *Erwinia rhapontici* During Growth in Host Plant Tissue

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*Erwinia rhapontici*가 기주식물 조직에서 생산한 Pectate Lyase의 특성

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ABSTRACT : *Erwinia rhapontici* causes soft-rot disease in a number of plants such as rhubarb, onion, hyacinth and garlic. Pectate lyase (Pel) depolymerizes pectin and other polygalacturonates, which is thought to play a role in bacterial invasion of plants. Pel activity was not detected in *E. rhapontici* cultured in a minimal salts medium containing glycerol, polygalacturonate, or citrus pectin as a carbon source. However, when sterilized potato tuber and Chinese cabbage slices were added to minimal salts polygalacturonate (0.5%) medium, *E. rhapontici* produced pectate lyase enzyme. Also Pel activity was consistently detected from macerated potato tubers, Chinese cabbage leaves, lettuce leaves and celery petioles tissue. Pel in the extract of macerated Chinese cabbage caused by *E. rhapontici* strain 1, resulted in electrolyte loss, tissue maceration and cell death of potato tuber tissue. These results indicate that *E. rhapontici* produces pectate lyase only in the presence of non-diffusible plant components, and that this enzyme probably contributes to its pathogenicity.

***Key words* : *Erwinia rhapontici*, pectate lyase, maceration, electrolyte loss, cell death.**

Since *Erwinia rhapontici* was first described from rhubarb by Millard (16), it was been reported from rhubard (15), onion (12, 18), garlic (7), hyacinth (22) and wheat (21). Although *E. rhapontici* caused rots of potato tuber, onion and cucumber slices, rot lesion is localized and advanced in a slow rate compared to that of *E. carotovora*. Differences between *E. carotovora* and *E. rhapontici* species are likely dependent on the production of pectinase as measured by pectate gel degradation at pH 7.0 (21). In some diseases, maceration is directly correlated with cellular death (10, 17, 25) and particularly, pectic-enzyme production is associated with the maceration and cell death of tissues, which is characteristic of soft-rot disease (9, 10).

E. carotovora subsp. *carotovora*, *E. carotovora* subsp. *atroseptica* and *E. chrysanthemi* are characterized by production of cell wall degrading enzymes such as pectate lyase (Pel), polygalacturonase, protease, cellulase and pectin lyase (6, 9). These enzymes secreted by the bacteria depolymerize the main constituents of the plant cell wall and can be detected both in tissues of infected plants and in liquid or solid culture media of the bacteria. Pel production by a bacterium does not necessarily confirm the pathogenicity (5, 23). A long-standing hypothesis concerning this observation is that pathogens are able to rapidly synthesize and secrete high amount of Pel in the presence of host tissues or pectic compounds (5, 23). Expression of pectic enzymes is important in soft-rot pathogenesis caused by *Erwinia* (9). Zuker and his colleagues showed that nonpathogenic bacteria synthesized pectic enzymes constitutively, whereas their synthesis in pathogenic bacteria was inducible (27, 28). In general, pectic enzyme synthesis is inducible by substrates such as pectate

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polymers and oligomers and isolated plant cell wall (8). Though *E. rhapontici* belongs to a soft-rot *Erwinia* group, this organism lacks in pectate degradation and gelatin liquefaction (13). At present, little is known about the Pel of *E. rhapontici*.

In this paper, the experiments were designed and conducted to understand the inducibility of Pel enzymatic activities of *E. rhapontici* after inoculation of the pathogen. Also I examined the consequent effects of the induced Pel activity on pathogenic maceration of plant tissues.

MATERIALS AND METHODS

Bacterial strains, media. The wild type *E. rhapontici*, *E. carotovora* subsp. *carotovora* and *E. chrysanthemi* strains in this study were obtained from Dr. Chatterjee, A. K. (University of Missouri). Cultures were maintained on Luria Bertani (LB) agar medium (14). For the determination of enzyme production, minimal salts (MS) medium (4) containing glycerol (0.5 g/ml). Polygalacturonic acid (PGA, 0.5 g/ml) and galacturonic acid (0.5 g/ml) was used.

Preparation of plant extract. Chinese cabbage leaves and potato tubers were washed with tap water and air dried at room temperature. Fresh plant tissues (100 g) homogenized with 100 ml of cold distilled water in a mixer for 2 min and in a blender for 10 min. Then the mixture was centrifuged 20 min at 10,000 rpm at 4℃ and the supernatant was filtered through a 0.45 μm milipore filter.

Plant maceration and enzyme extraction. The ability of bacterial strains to macerate plant tissues was tested using detached parts of six different plants, including slices of potato tubers, celery petioles, Chinese cabbage leaves, lettuce leaves, and onion bulbs. The detached parts were treated with 10% Chlolex for 10 min and rinsed with tap water for 20 min and then air dried at room temperature. Sterilized plant materials were cut into 5~10 cm slices, and inoculated with bacterial suspensions containing 10^8 colony-forming units per mililiter. Twenty μl of bacterial suspension was placed in the center of tissue sections on moistened filter papers in petri plates. Plates were incubated in plastic boxes lined moistened paper towels for 5 day at room temperature. The extent of tissue maceration was determined by measuring the wet weight of the macerated tissue. To obtain crude extracts from macerated tissues, macerated tissues were removed from the infected slices and centrifuged at 10,000 rpm for 30 min at 4℃. The crude extract was dialysed against 2,000 ml of a buffer solution containing 10 mM Tris-HCl (pH 7.0) for 8 hr at 4℃.

The crude enzyme of *E. chrysnthemi* strain 16 was obtained from an overnight (18 hrs) culture at 28℃ in minimal salts-casamino acid (1%) medium containing 0.2% pectate. The culture supernatant containing ca. 100 unit/ml of Pel activity was dialysed against 4,000 ml of 10 mM Tris-HCl (pH 7.0) for 4 hrs.

Enzyme assays. Bacterial cultures were harvested (10,000 rpm for 20 min at 4℃) and the supernatants were stored at 4℃. Pel was assayed according to Starr *et al.* (23) in a 0.6 ml reation mixture contained 0.25 ml of a 5.75 mg/ml concentration of sodium polygalacturonate, 0.25 ml of 0.78 mM $CaCl_2$ in 230 mM Tris (pH 8.5), and 0.1 ml of crude enzyme or water. The rate of reaction at 30℃ was measured by a Simazu spectrometer at 235 nm. One unit of Pel activity is defined as the amount of the enzyme that increases 1.0 of 235 nm absorbance per minute at 30℃.

Measurement of cell death, tissue maceration, permeability. Cell death and tissue maceration were estimated by the method of Basham and Bateman (1, 2). At 1 hr intervals, duplicate potato disks (10 mm×1 mm) were placed in 1 ml of 20 mM phosphate buffer (pH 7.5) containing 600 mM KNO_3, 1 mM $CaCl_2$ and 0.01% Neutral Red for 20 min and then rinsed with 3 ml of the KNO_3 solution without Neutral Red.

Cell death was observed visually, based on the ability of cells to accumulate and retain Neutral Red. Degree of dye accumulation was measured in the range 0 (complete accumulation) to 5 (none).

Maceration of *E. rhapontici* and *E. chrysanthemi* crude enzyme-treated disks was estimated after the disks were stained with Neutral Red. Base on the easiness with which the tissue could be punctured with a needle, the degree of cohesion of cells in the tissue disks was estimated.

Changes in the permeability of potato disks was measured by determining the loss of electrolytes from potato disks during treatment. Reaction mixture was consisted of 10 mM Tri-HCl buffer (pH 8.5) containing 10 ml of 6 units Pel, 1 mM $CaCl_2$, and streptomycin sulfate and spectinomycin (100 mg/ml,

each) (11). Potato disks were placed in an 100 ml beaker with the above mixture, and shaken on a rotary shaker at 140 cycles/min at 28℃, Five disks were removed from the reaction mixtures at 1 hr intervals, rinsed with distilled water, placed in 15 ml of distilled water and mixed gently for 10 sec. Conductivity was measured with a conductivity meter (Yellow Springs Instrument Co). The mixture was kept at room temperature for 10 min and then conductivity was measured after gentle mixing for 10 sec; this represented the final readings. Corrected values (μmhoms/min) were obtained by subtracting the initial reading from the final reading (11). Tissue containing autoclaved enzyme was used as a control.

RESULTS

Tissue maceration by wild type strains and the presence of pectate lyase in macerated tissue. *E. rhapontici* (Er1) strains caused rotting (maceration) of potato tubers, Chinese cabbage leaves, lettuce leaves, celery petioles, and onion bulbs (Table 1). Because Er1 causes only a slow, weak and erratic rot of hosts, this bacterium is different from *E. carotovora* subsp. *carotovora* (Ecc71).

The activities of extracellular enzymes of rotted plant tissues are given in Table 2. Pel was detected in rot extracts caused by Er strains but the activities were lower than those caused by Ecc71. In order to determine if the activity of pel is due to the induction by some compound in the plant, Pel activity was examined after incubation of Ecc71 and Ec16 on the MS containing the extract of Chinese cabbage. Er strains did not produce Pel in artifical media containing glycerol, pectin, polygalacturonate or water extracts of Chinese cabbage leaves, potato tubes, but Pel was detected in a 0.5% polygalacturonate MS medium containing potato tuber (Table 3).

Time-course of tissue maceration caused by Er strain and Pel production in soft-rot extracts of infected tissues were measured (Fig. 1). When Er1 was inoculated onto Chinese cabbage leaves, Pel activity was detected in the crude tissue extract 36 hrs after inoculation and increased as maceration continued. In contrast, tissue maceration and Pel activity caused by Ecc71 were observed at 24 hr after inoculation. Pel activitiy was increased within 48 hr after inoculation, and deceased rapidly afterward. But tissue maceration was increased throughout the incu-

Table 1. Tissue maceration of various vegetables by *E. rhapontici* and *E. carotovora* subsp. *carotovora* strains

Strain	Potato tuber	Celery petiole	Onion bulb	Chinese cabbage leaf	Lettuce leaf
Er1[a]	+[b]	+	+	+	+
Er102	+	+	+	+	+
Er109	+	+	+	+	+
Er112	+	+	+	+	+
Ecc71	+++	+++	++	+++	++

[a] Er: *Erwinia rhapontici*, Ecc: *E. carotovora* subsp. *carotovora*.
[b] +: Slightly macerated, ++: moderately macerated, +++: highly macerated.

Table 2. Pectate lyase activities in plant macerated tissues macerated by *E rhapontici* strain 1 (Er1) and *E. carotovora* subsp. *carotovora* strain 71 (Ecc71)

Bacterial strain	Source of enzyme	Activity (unit/ml)
Ecc71	Potato rot	21
	Chinese cabbage rot	9
	MS[a]+pectate medium	15
Er1	Potato rot	11
	Chinese cabbage	5
	MS+glycerol	ND
	MS+pectin	ND
	MS+pectate	ND

[a] MS: Minimal salts, [b] ND: Not detected.

Table 3. Levels of pectate lyase activity produced by *E. carotovora* supsp. *carotovora* strain Ecc71, *E. chrysanthemi* strain Ec16 and *E. rhapontici* strains (Er) in a media containing pectate, Chinese cabbage extracts or potato tuber as carbon sources

Bacterial strain	Pectate lyase activity (unit/ml)			
	Pectate[a] medium	Chinese cabbage extract	Potato tuber extract	Potato tuber
Ecc71	5.1	2.4	1.0	1.8
Ec16	8.9	5.8	4.3	2.5
Er1	ND[b]	ND	ND	1.5
Er102	ND	ND	ND	1.3

[a] Minimal salts medium containing 0.2% polygalacturonate.
[b] ND: Not detected.

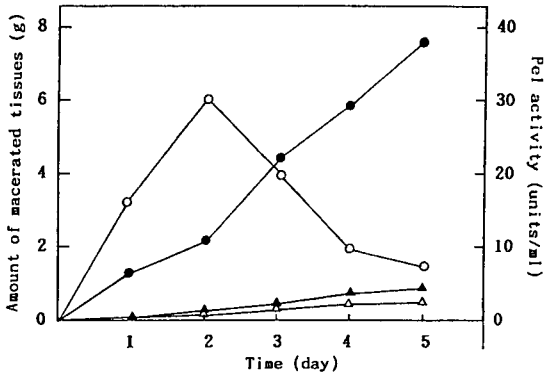

Fig. 1. Time course of Chinese cabbage maceration and pectate lyase (Pel) production by *E. rhapontici* strain 1 and *E. carotovora* supsp. *carotovora* strain 71. ●–● maceration (Edd71), ○–○ Pel (Ecc71), ▲–▲ maceration (Erl), △–△ Pel (Erl).

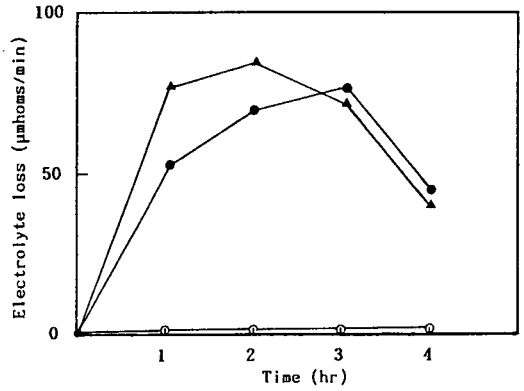

Fig. 2. Electrolyte loss in potato tuber discs treated with Pel extracted from Chinese cabbage rot elicited by *E. rhapontici* (Erl) or Pel present in culture supernatant of *E. chrysanthemi* (Ec16). ●–● Erl, ▲–▲ Ec16, ○–○ Autoclaved Pel (Erl).

bation time.

Biochemical properties and tissue maceration ability of pectate lyase in rot extracts. Leakage of eletrolytes was estimated by measuring conductivity changes. Electrolytic leakages in the control using autoclaved enzyme were negligible (Fig. 2). For potato tuber disks treated with crude extract, the rates of conductivity increased very rapidly. Maximum loss was occurred in potato tuber tissue 2 hr after the addition of Ec16 Pel, and decreased slightly during the rest of the incubation time. But the maximum loss in Erl occurred 3 hrs after the addition.

Cell death was determined with the Neutral Red

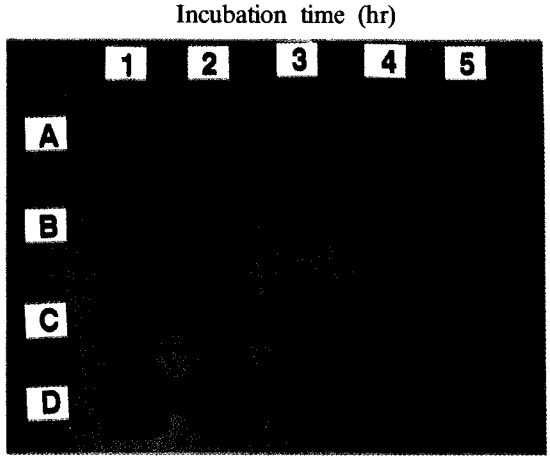

Fig. 3. Cell death of potato tuber discs treated with Pel extracted from Chinese cabbage rot induced by *E. rhapontici* (Erl, Erl02) or Pel present in culture supernatant of *E. chrysanthemi* (Ec16). Potato discs were stained with neutral red followed by destained with phosphate buffer containing 0.6 M KNO_3 and $CaCl_2$. Autoclaved Pel (Erl) was used as a control. A: Erl, B: Erl02, C: Ec16, D: Autoclaved Pel.

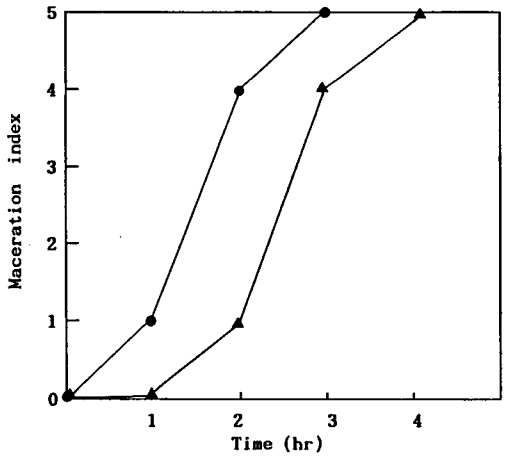

Fig. 4. Cell maceration of potato tuber discs treated with Pel extracted from Chinese cabbage rot elicited by *E. rhapontici* (Erl) or Pel present in culture supernatant of *E. chrysanthemi* (Ec'6). Degree of cell separation was examined by scratching totato disc surface by using a needle. 0: No cell separation, 5: Complete cell separation. ▲–▲ Erl, ●–● Ec16.

procedure (1, 2). Cell death of Ec16 Pel reation mixture was approximately two times higher in 3 hr when compared to that of Pel Erl and Erl02 Pels (Fig. 3). Tissue maceration occurred in potato tuber

tissue 2 hrs after addition of Er1 Pel. Tissue maceration of potato disks occurred rapidly and resulted in death after 4 hrs. Ec16 Pel was much more effective in inducing these changes (Fig. 4). Tissue maceration occurred in the similar pattern shown in cell death experiments. Cellular death and maceration paralleled each other, but electrolye loss preceded maceration and death.

DISCUSSION

The potential role of cell wall hydrogenases from potato tuber and Chinese cabbage tissue in maceration by *E. rhapontici* was investigated. *E. rhapontici* did not utilize ploygalacturonate as single carbon source nor produced Pel when grown in the medium containing polygalacturonate, pectin, galacturonate and water extract of host plants. This suggests that this organism apparently lacks the catabolic enzymatic system for the polysaccharides provided in an artificial medium. Thus, it reasonable to argue that Pel produced by the pathogen has a function to make the nutrients available to itself within host cells.

Plant extracts did not show any enzymatic activity of Pel. Also there was no detectable enzymatic activity in *E. rhapontici* grown in medium containing a wide range of dicotyledonous plant extracts. However, this experiment showed that Pel activity was able to be induced in the macerated plant tissue only after inoculation of *E. rhapontici*. Therefore, it is highly possible that intacts host plants contain Pel inducing materials. This result strongly suggest that Pel inducing factor(s) may exist in plant tissues as non-diffusible form(s).

These data show that Pel from Er1 in Chinese cabbage caused tissue maceraiton and resulted in cell death of potato tubers. Another striking effect of Pel is increase of the membrane permeability of potato disks. Therefore, Pel is probably important in the development of tissue maceration in host plants. Because of the maceration and cell death by Pel, this enzyme has been thought to play a role in the soft-rot disease caused by *E. rhapontici*. Even though enzymatic maceration of plant tisue is an important process to plant pathogenicity in soft rot *Erwinia* (3, 6), it remains to ask whether Pel is a significant factor for pathogenicity in *E. rhapontici*. Pel synthesis by *E. carotovora* subsp. *carotovora* and *E. chrysanthemi* was induced by the degradation products of pectins regardless of the presence of intact plant materials (20, 21), and was also stimulated by certain plant extracts (24, 26).

While Pel has been extensively studied in *E. carotovora* subsp. *carotovora* and *E. chrysanthemi*, information concerning their role in the pathogenicity of *E. rhapontici* is extremly limited. The latter organism is much more restricted in host range than *E. carotovora* subsp. *carotovora*. A study to determine whether the differences in enzymes synthesis are associated with the pathogenicity or the different range of host plant will be significant to understanding a mechanism underlying Pel and host-specific pathogenicities. Further study for the genetics and physiology of these enzymes involved in the pathogenicity will contribute to understand their role for the induction of plant disease.

요 약

*Erwinia rhapontici*는 rhubarb, 양파, 히야신스, 마늘 같은 작물에 무름병을 일으킨다. Pectate lyase(Pel) 효소는 pectin과 polygalacturonate의 중합체를 분해하며 세균의 식물 감염에 관여한다. Glycerol, polygalacturonate, 감귤 pectin이 포함된 minimal salts 배지에서 배양된 *E. rhapontici*는 Pel 효소활성이 검출되지 않았다. 그러나, 0.5% plygalacturonate minimal salts배지에 표면살균한 감자 괴경이나 배추 조각을 가해여 배양할 경우는 Pel을 생산하였다. 또한, Pel 활성은 이 균에 의해 붕괴된 감자괴경, 배추잎, 상치잎, 세러리 엽병 조직에서도 검출되었다. *E. rhapontici*에 의해 붕괴된 조직에서 추출된 Pel은 감자괴경 조직으로부터 전해질을 유출시켰고, 감자괴경의 조직붕괴 및 cell death를 일으켰다. 이러한 결과에서 *E. rhapontici*는 물에 용해되지 않는 식물 성분이 존재할 때만 Pel을 생산하고, Pel 효소 병원성 발현에 기여할 것으로 시사된다.

ACKNOWLEDGEMENT

Auther thanks Dr. A. K. Chatterjee for providing bacterial strains.

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