Production of Pectin Lyase by *Colletotrichum lindemuthianum* in Culture and in Infected Bean (*Phaseolus vulgaris*) Tissue

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Colletotrichum lindemuthianum race γ secreted two forms of pectin lyase, having pI values of 8.2 and 9.7, when grown in culture with sodium polypectate or isolated *Phaseolus vulgaris* hypocotyl cell walls as the main source of carbon. A single form of polygalacturonase was also present in both media. A pectin lyase, with a pI value of 9.7 was detected in necrotic *P. vulgaris* tissue infected with race γ , but polygalacturonase activity was absent.

INTRODUCTION

Colletotrichum lindemuthianum (Sacc. et Mgn.) Bri. et Cav. produces large water-soaked lesions on susceptible cultivars of bean, *Phaseolus vulgaris* L. It is a hemibiotrophic pathogen, whose infection hyphae exhibit an initial period of biotrophic growth, when they grow through plant cell walls and obtain nutrients for growth without killing the infected cells. The initially infected cells eventually die, and the fungus then grows extensively in the dead tissues (necrotrophy), which become extensively macerated as lesions become evident (Bailey, 1982). The present research programme aims to assess the involvement of cell wall-degrading enzymes in both biotrophic and necrotrophic fungal growth and to determine whether changes in enzyme secretion during infection can explain the transition from biotrophy to necrotrophy.

Cell wall-degrading enzymes play a major role in symptom development in many diseases (Bateman & Basham, 1976) and several, including polygalacturonase, α -galactopyranosidase, α -arabinofuranosidase, cellulase, β -xylosidase and protease have been described from *C. lindemuthianum* (English *et al.*, 1971; Anderson & Albersheim, 1972; Ries & Albersheim, 1973; Barthé *et al.*, 1981; Cervone *et al.*, 1981). However, except for a brief comment by Cervone *et al.* (1981), there are no reports of the production of pectin lyase by this pathogen, nor are there any data concerning the presence of any cell wall-degrading enzymes in bean tissues infected by this pathogen.

This paper describes the properties and activity of pectin lyase produced by the race γ of *C. lindemuthianum* in different media and in lesions of infected hypocotyl tissue of *P. vulgaris* cv. Kievitsboon Koekoek, with which race γ is compatible.

METHODS

Preparation of cell walls of Phaseolus vulgaris. Seedlings of *P. vulgaris* cv. Kievitsboon Koekoek were grown in moist vermiculite in a growth cabinet (Fisons 9600) at $25 \pm 0.5^\circ\text{C}$, 80% relative humidity, $40 \mu\text{E m}^{-2} \text{s}^{-1}$ photon flux density, 16 h photoperiod. The seedlings were harvested 10 d after sowing, following a period of 36 h in complete darkness. The hypocotyls were frozen and cell walls isolated according to the method described by English *et al.* (1971), except that 500 mM-potassium phosphate buffer was used for extraction instead of 100 mM-buffer, to minimize the presence of ionic proteins (Jones *et al.*, 1972). The yield of cell walls was approximately 2.5% of tissue fresh weight. Starch was not detected in the isolated cell wall preparation by an iodine test.

Fungal culture and preparation of nutrient media. Race γ of *Colletotrichum lindemuthianum* (Rowell & Bailey,

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Abbreviations: PL, pectin lyase; TBA, thiobarbituric acid.

1983), was grown in an ammonium tartrate medium (Byrde & Fielding, 1968) containing sodium polypectate or isolated cells walls (0.5 g l^{-1}) as its main source of carbon. All the media were autoclaved for 15 min at 121°C .

One litre glass 'penicillin' flasks containing 250 ml medium were inoculated with 1 ml of a suspension of spores (containing about 10^6 spores), obtained from 7 to 8-d-old sporulating cultures of *C. lindemuthianum* grown as described by Bailey & Deverall (1971). The inoculated media were incubated without shaking at $17 \pm 1^\circ\text{C}$, under a 'black' fluorescent light (F 8T5, GTE Sylvania Inc., Lighting Products, Denver, Colo., USA) with a 12 h photoperiod.

The cultures were harvested 12 d after inoculation by filtering through Whatman no. 1 filter paper. The filtrate was dialysed against distilled water for 48 h at 4°C , and was concentrated ($\times 10$) by freeze drying.

Inoculation of the seedlings. Seedlings of *P. vulgaris* cv. Kievitsboon Koekoek were grown for 7 d in the illuminated growth cabinet described above. The hypocotyl segments were inoculated with *C. lindemuthianum* and incubated at 16°C (Rowell & Bailey, 1983).

Extraction of enzymes from infected plant material. Seven to eight days after inoculation, brown water-soaked lesions were visible on the hypocotyls. At this stage, the entire hypocotyls were harvested, frozen immediately, and freeze dried. The dry tissue was extracted and prepared for iso-electric focusing as described by Fielding (1981), although 1.0 M-NaCl was used instead of 0.1 M-KCl , since in preliminary experiments, extracts prepared with this reagent gave greater enzyme activity.

Iso-electric focusing. Iso-electric focusing was carried out in an LKB 110 ml column using LKB ampholines of pH range 3.5–10.0, in a linear sucrose gradient at 10°C for 48 h (Laborda *et al.*, 1973). The column was eluted and 3.0 ml fractions were collected using a peristaltic pump at 120 ml h^{-1} . The pH of the fractions was recorded immediately afterwards and the enzyme activity of the fractions was measured.

Enzyme assays. Pectin lyase (PL; EC 4.2.2.10) activity was measured spectrophotometrically (Byrde & Fielding, 1968). The standard reaction mixture comprised 3.0 ml 0.25% (w/v) citrus pectin in 0.1 M-Tris/HCl buffer of appropriate pH and 0.1 ml enzyme solution. The absorbance at 240 nm was read immediately after the addition of the enzyme sample and after an incubation period at 30°C . One unit of enzyme activity is defined as that amount which produces $1 \mu\text{mol}$ unsaturated uronide in 1 min, based on the molar absorption for the product of 4600 (Hislop *et al.*, 1979). PL activity was also examined using the thiobarbituric acid (TBA) method (Ayers *et al.*, 1966). After specified reaction periods, 1.0 ml samples of the reaction mixture described above, were added to 6.0 ml TBA reagent ($5.0 \text{ ml } 0.01 \text{ M-TBA}$ and $1.0 \text{ ml } 1 \text{ M-HCl}$), and kept in a boiling water bath for 60 min. After cooling, the absorption was read at 550 nm.

Polygalacturonase (EC 3.2.1.15) was estimated by the cup-plate assay method of Dingle *et al.* (1953) using a sodium polypectate-agar gel. Activities are expressed relative to an aqueous solution (1 mg ml^{-1}) of Pectinol 10M which was defined as having 100 units of polygalacturonase activity ml^{-1} .

The polygalacturonase activity in extracts of infected lesions was also determined by measuring the decrease in viscosity of a 1% (w/v) aqueous solution of sodium polypectate at pH 5.0, using a $0.1 \text{ M-sodium acetate}$ buffer in an Ostwald viscometer at 25°C (Fielding & Byrde, 1969).

Molecular weight determinations. Molecular weights were determined as described by Andrews (1964), using a column of Sephadex G-150 or Sephadex G-100, eluted with Tris/HCl buffer (0.05 M , pH 7.6), plus 0.1 M-KCl and calibrated with the following marker proteins: serum albumin (both monomer and dimer in Sephadex G-150), egg albumin, trypsin inhibitor and cytochrome c.

Effect of heat and pH on enzyme activity. Heat inactivation was studied by placing enzyme samples (5 ml) in test-tubes maintained in a water bath at desired temperatures for 10 min. The tubes were cooled rapidly in cold water (12°C) and the contents then assayed for enzyme activity (Spalding & Abdul-Baki, 1973). Borate, Tris and phosphate buffer systems were used to determine the optimum pH for enzyme activity.

Chemicals. Sources were as follows: citrus pectin, H.P. Bulmer Ltd, Hereford, UK; polygalacturonic acid and the marker proteins, Sigma; sodium polypectate, Exchange Lemon Product Co., California, USA; Pectinol 10M, Rohm & Haas Ltd, Philadelphia, USA. All other chemicals were of at least 'Laboratory Reagent' grade and, when available, 'Analar' chemicals were used.

RESULTS

Enzyme activity in culture filtrates

Filtrates from 12-d-old cultures of *C. lindemuthianum* grown with sodium polypectate as the main carbon source had PL activity. When the culture filtrate was electrofocused (Fig. 1*a*), two distinct peaks of activity were present. A minor peak was present at pI 8.2 (PL I) and the major peak at pI 9.7 (PL II).

PL activity was also present in filtrates from cultures where cell walls isolated from hypocotyls of *P. vulgaris* were the main sources of carbon. Iso-electric focusing again produced two peaks of

Fig. 1. Iso-electric focusing of culture filtrates of *Colletotrichum lindemuthianum*. (a) Sodium polypectate medium; (b) cell wall medium; and (c) extracts of lesions from infected bean hypocotyls. —, pH gradient; ●, PL activity measured as change in absorbance at 240 nm.

enzyme activity (Fig. 1*b*). In these filtrates, however, the major peak of activity was at pI 8.2 (PL I), with much less activity at pI 9.7 (PL II).

Culture filtrates from both media also showed a single peak of polygalacturonase activity at pI 9.3 (not shown). Lyase activity was also detected in both culture filtrates when polygalacturonic acid was used as the substrate in place of citrus pectin.

Effect of calcium ions on pectin lyase activity

CaCl₂ was added to reaction mixtures to give concentrations between 0 and 0.06 M (Cooper *et al.*, 1978), and the activity of PL I and PL II, i.e. those obtained from iso-electric focusing, was measured. Neither of the forms had an absolute requirement for calcium ions for activity, but their activity increased with the increase in calcium ion concentration. PL I from both media showed maximum activity at 0.001 M and the activity decreased thereafter. PL II had its maximum activity at 0.05 M-calcium ion concentration. PL II seemed more calcium dependent than PL I. Both forms of the enzyme isolated from the sodium polypectate medium showed a greater increase in activity with the increase in calcium ion concentration than their respective forms produced in the cell wall medium.

Effect of pH and temperature inactivation

All the forms of PL showed maximum activity under alkaline conditions. PL I from sodium polypectate or cell wall medium had its optimum at pH 9.3 or at pH 8.8, respectively. PL II from both media had optimum activity at pH 9.8 (Fig. 2*a*).

Treatment of the enzyme at 30 °C for 10 min increased its activity slightly compared with that at 20 °C, but at temperatures higher than 30 °C activity was reduced. At 60 °C, activity of all forms of the enzyme showed a decrease of nearly 75%, although some activity remained even after treatment at 70 °C.

Fig. 2. Effect of pH (a) and temperature (b) on the activity of the two forms of PL from a sodium polypectate culture filtrate. ○, PL I; ●, PL II. PL activity was measured using change in absorbance at 240 nm. In both instances a similar pattern of activity was also obtained for the two forms of PL from the cell wall medium.

Enzyme activity in infected necrotic tissue

Iso-electric focusing of extracts of lesion tissue in bean hypocotyls infected with race γ produced a single peak of PL activity, focusing at pI 9.7, with a shoulder in the pI range 7.9 to 8.5 (Fig. 1c). No PL activity was detected in extracts of healthy *P. vulgaris* hypocotyl tissue.

Polygalacturonase activity could not be detected in the extracts of lesions either by the plate assay or by viscometry. In the plate assay of the iso-electrically focused fractions from extracts, white opaque rings developed between pI 8.0 and 10.5 instead of the usual clear rings obtained with similar fractions from culture filtrates. After autoclaving at 121 °C for 15 min, the fractions from the extracts still produced the opaque rings, whilst no rings were produced by autoclaved fractions from the culture filtrate.

Molecular weights of the multiple forms of pectin lyase

The molecular weights of the forms of the enzyme from the culture filtrates were determined in two experiments using the Sephadex G-150 column. PL I from the sodium polypectate medium had a mean molecular weight of 23000, whilst the molecular weight of PL I from the cell wall medium was 23500. For PL II the estimated mean molecular weights from sodium polypectate and the cell wall medium, were 28500 and 27000, respectively. The molecular weight of the PL II (pI 9.7) from tissue extracts was determined using the Sephadex G-100 column, as 24500.

DISCUSSION

Colletotrichum lindemuthianum secreted two forms of pectin lyase (PL I and PL II) in artificial media. Many plant pathogenic micro-organisms produce multiple forms of individual types of pectolytic enzymes (Hancock, 1976; Byrde, 1982), and the presence of multiple forms of an enzyme could confer an added advantage to the pathogen, because some forms may be more stable in the host than others (Byrde & Archer, 1977).

On the basis of the characters studied, i.e. molecular weights, pH activity, heat stability, calcium dependency and iso-electric points, the fungus seems to secrete the same two forms of the enzyme when growing on either pectate or on isolated cell walls as the main carbon source. However, the relative amounts of the two forms obtained from the two media differed. At the time of harvesting PL I was the major form from the cell wall medium, whereas PL II was the major form from the sodium polypectate medium. Though the activity of all the forms was stimulated by calcium ions, this enhancement was less pronounced in the forms from the cell

wall medium. This could be attributed to the presence of calcium ions in isolated cell walls (Cooper *et al.*, 1978) which may reduce the effect of added calcium.

One form of pectin lyase (PL II) was predominant in necrotic lesions, although there were indications of very low PL I activity.

The inability to detect polygalacturonase activity in extracts of lesion tissue contrasts with the high PL activity therein and with the presence of both enzymes in culture filtrates. This finding needs an explanation; it also emphasizes that PL is more likely to be of importance in pathogenesis especially during necrotrophic pathogen growth. Further studies will assess the regulation of PL activity during the biotrophic stage of the disease and during the transition from biotrophy to necrotrophy.

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