Multiple Forms of Polygalacturonase in Apple and Carrot Tissue Infected by Isolates of Botrytis cinerea

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Isoelectric focusing of extracts of carrot root and apple fruit parenchyma infected by each of three isolates of Botrytis cinerea showed the presence of forms of endopolygalacturonase (PG) with isoelectric points (pI) of about 4.5 (a minor peak) and 8.3–8.8. One isolate gave the hitherto unreported peak of pI 8.8 in both tissues, whereas the other two isolates gave a peak of 8.3. The molecular weights of the PGs of pI 8.3–8.8 were estimated to be about 30000. The variation in pI probably reflects the variability of B. cinerea. No evidence of specific enzymic adaptation to host tissues was shown by the ability of PGs to cause changes in permeability of carrot and apple parenchyma.

INTRODUCTION

The plant pathogen Botrytis cinerea has long been known to secrete pectolytic enzymes (Brown, 1915), and there have been several reports of the production of multiple forms of polygalacturonase (EC 3.2.1.15) by the fungus (Zalewska-Sobczak & Urbanek, 1975; Drawert & Krefft, 1978; Magro et al., 1980; Fielding, 1981). The fungus is recognized as having considerable natural phenotypic variability (e.g. Grindle, 1979), and this may be reflected in the different patterns of multiple forms described.

This paper describes multiple endopolygalacturonase (PG) forms present in extracts of two host tissues infected by each of three isolates of the pathogen.

METHODS

Production of PG in host tissues. Apple fruits (Malus communis Lam. cv. Golden Delicious) and carrot roots (Daucus carota L. cv. Chantenay) were washed with tap water, surface sterilized by immersion in 1% (w/v) sodium hypochlorite for 10 min and rinsed with sterile water. Discs (6 mm diam.) of three isolates of Botrytis cinerea (Magro et al., 1980), from seven-day-old cultures on potato-dextrose agar were introduced into holes (1.5 cm deep) made on apples and carrots with a cork borer. Symptoms began to be visible around the inoculation point as brown areas 24–48 h later. Visibly rotted tissues and 1 cm of the surrounding zone not yet affected by the rot were removed 3 and 5 d after the inoculation. Infected tissues were gently ground in a mortar and to them were added cold 0.05 M-Tris/HCl buffer pH 7.8 [1 ml (g tissue)−1] containing 0.1 M-KCl and 0.5% (w/v) cysteine. After 30 min at 4 °C, the mixtures were strained through three layers of cheese-cloth, centrifuged at 4 °C (20000 g for 20 min) and exhaustively dialysed against several changes of distilled water at 4 °C. The crude enzyme preparations were frozen at −20 °C until used.

Enzyme purification. Crude enzymes were subjected to isoelectric focusing (IEF) in a 110 ml LKB 8100 apparatus over the range pH 3–10 using ampholine carriers in a glycerol gradient (0–60%, w/v). The cathode (2.5 ml 1 M-NaOH and 7.5 ml H2O) was at the top and the anode (4 ml 1 M-H2PO4, 12 ml H2O and 15 g sucrose) at the base of the column. Voltage was gradually raised to 1000 V and maintained until the end of the run (48 h). Fractions (2 ml) were collected and the pH and PG activity of each fraction were determined.

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Abbreviations: IEF, isoelectric focusing; NaPP, sodium polypectate; PG, endopolygalacturonase; VU, viscometric units.

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The fractions of the main peak of PG activity of each host-fungal isolate combination were pooled, exhaustively dialysed against distilled water and concentrated against polyethylene glycol (Serva Feinbiochemica) to half the initial volume. For molecular weight estimations, samples of about 1 ml were subjected to gel filtration in a column (1.6×40 cm) of Sephadex G-75 coarse (Pharmacia) equilibrated with 0.05 M-sodium phosphate buffer pH 7.0, containing 0.1 M-KCl and 0.02% (w/v) sodium azide. Elutions were carried out with the same buffer at a flow rate of 0.5 ml min⁻¹. Blue Dextran (mol. wt 2000000), bovine serum albumin (mol. wt 67000), egg albumin (mol. wt 45000), chymotrypsinogen A (mol. wt 25000), horse myoglobin (mol. wt 17800) and potassium chromate (mol. wt 194) were the reference markers. The fractions (1.5 ml each) with PG activity were pooled, dialysed and concentrated as previously described and used for determining percentage hydrolysis and permeability changes.

Polygalacturonase activity assays. The cup-plate assay technique described by Dingle et al. (1953), with sodium polypectate (NaPP; Sunkist Growers) as substrate, was used for a screening of fractions from IEF and Sephadex columns. Activities were expressed relative to an aqueous solution (0.5 mg ml⁻¹) of pectinase (Sigma) defined arbitrarily as having 100 units activity ml⁻¹.

More accurate determinations were obtained with the viscometric method, using size 200 Cannon Fenske viscometers at 30 °C. Reaction mixtures consisted of 3 ml 1 % (w/v) NaPP in 0.05 M-sodium acetate buffer pH 4.6, samples of enzyme preparation and buffer to a final volume of 6 ml. Viscometric units (VU) of enzyme activity were determined by dividing 1000 by the number of minutes required for 1 ml enzyme to reduce the relative viscosity by 50 %. These same reaction mixtures were assayed for reducing groups by Nelson’s method (1944), at 30 °C.

Changes in permeability induced by the action of the main peaks of PG activity on healthy apple and carrot tissues were measured by determining the loss of K⁺ ions. Disks (5 mm diameter, 2 mm thick) obtained from apple fruits and carrot roots, were weighed, repeatedly washed in distilled water and rinsed with 0.05 M-sodium acetate buffer pH 4.6 for 15 min. Reaction mixtures, in 25 ml Erlenmeyer flasks, contained 10 discs, samples of enzyme (200 VU) and 0.05 M-acetate buffer pH 4.6 to a final volume of 5 ml. Water blanks without enzyme caused very little leakage from discs of either the apple or carrot. Flasks were slowly shaken at 30 °C and at 10 and 20 min tissues were removed and the liquid was tested for K⁺ ions with a Radiometer selective ion electrode F2312K. Data are expressed as μg K⁺ ions (g fresh tissue)⁻¹ on the basis of a calibration curve with standard KCl solutions in 0.05 M-acetate buffer pH 4.6. Values are the difference between treatments and controls without enzyme in at least three replicate experiments.

RESULTS AND DISCUSSION

The polygalacturonase patterns produced by isoelectric focusing of extracts of carrot tissue infected by isolates BB, H and PP, respectively, are shown in Fig. 1 (a–c). Corresponding data for infected apple tissue are presented in Fig. 2 (a–c). There was a close similarity in the focusing patterns for a given isolate when infecting either host tissue; moreover, there was little difference between isolates H and BB. The main PG form had a pI of 8.3, with minor peaks at about 4.5. Isolate PP, by contrast, showed the presence of a PG peak with pI 8.8 in extracts of both tissues, this difference probably reflecting the variability of the pathogen (cf. Fielding, 1981) particularly as all three isolates originated from vines in a single Italian region (Magro et al., 1980). With Monilinia laxa grown in vitro, by contrast, Willetts et al. (1977) noted differences in pectin lyase patterns only among Australian isolates, and Fielding (1981) reported little variation in the major PG in extracts of tissue infected by M. laxa.

The pI 8.8 peak from B. cinerea was not detected in earlier studies in vitro (Magro et al., 1980). Extracts of other isolates showed quantitative enzyme differences, many of the PG forms detected in vitro not being found, demonstrating either changing of pI by binding with host products or selective secretion in the host.

Table 1 summarizes the isoelectric points, activity, reducing group release and estimated molecular weights of the main PG peak found in each extract after purification by gel-filtration. The PG secreted by B. cinerea in infected tissue is an ‘endo’-enzyme, since the percentage of bonds hydrolysed at 50 % loss of viscosity was only 1–2%. This confirms earlier findings (Drawert & Krefft, 1978; Magro et al., 1980). It is not clear if the small differences obtained in molecular weight estimates were significant. The values obtained are typical of many extracellular rhamnogalacturonan-degrading enzymes, e.g. Garibaldi & Bateman (1971); Byrde & Willetts (1977).
B. cinerea polygalacturonase in infected tissues

Fig. 1. Isoelectric focusing of polygalacturonases in extracts of carrot tissue infected by isolates BB (a), H (b) and PP (c). Ampholine carriers pH 3 to 10; 2 ml fractions were collected. ●—●, PG, assayed by the cup-plate method; ———, pH gradient.

Fig. 2. Isoelectric focusing of polygalacturonases in extracts of apple tissue infected by isolates BB (a), H (b) and PP (c). Ampholine carriers pH 3 to 10; 2 ml fractions were collected. ●—●, PG, assayed by the cup-plate method; ———, pH gradient.

Table 1. Characteristics of the main peaks of PG activity after isoelectric focusing and subsequent purification involving gel filtration

<table>
<thead>
<tr>
<th>B. cinerea isolate/host</th>
<th>pI</th>
<th>VU ml⁻¹*</th>
<th>Hydrolysis† (%)</th>
<th>Mol. wt‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>H/apple</td>
<td>8-3</td>
<td>55</td>
<td>1.4</td>
<td>25000</td>
</tr>
<tr>
<td>H/carrot</td>
<td>8-3</td>
<td>2500</td>
<td>1.5</td>
<td>32000</td>
</tr>
<tr>
<td>BB/apple</td>
<td>8-3</td>
<td>60</td>
<td>2.0</td>
<td>26000</td>
</tr>
<tr>
<td>BB/carrot</td>
<td>8-3</td>
<td>256</td>
<td>1.1</td>
<td>24500</td>
</tr>
<tr>
<td>PP/apple</td>
<td>8-8</td>
<td>33</td>
<td>1.4</td>
<td>26000</td>
</tr>
<tr>
<td>PP/carrot</td>
<td>8-8</td>
<td>35</td>
<td>1.6</td>
<td>27000</td>
</tr>
</tbody>
</table>

* Viscometric units (VU): 1000/t, where t = time in min for 50% viscosity reduction of NaPP solution.
† Reducing groups formed (at the time of 50% loss in viscosity of NaPP solution), 100 being those that would be formed if all the substrate was hydrolysed.
‡ Molecular weight was determined by gel filtration (see Methods).

Table 2 shows the leakage of K⁺ from healthy tissues exposed to equal activities of the partially purified PG (pI 8-3 or 8-8) extracted from host tissues. The PGs obtained from infected apple caused more rapid K⁺ leakage than did those from the infected carrot tissues irrespective of the healthy tissue being treated, for isolates BB and H; with the PGs of isolate PP (pI 8-8) by
Table 2. Loss of K⁺ ions [μg (g fresh wt)⁻¹] from healthy apple tissue (a) and carrot tissue (b) after treatment with pI 8.3 or 8.8 polygalacturonases

Enzymes (200 VU) were obtained from apple and carrot tissues infected with isolates H, BB and PP of *Botrytis cinerea*. Means followed by different letters are significantly different at *P* = 0.1 by Duncan’s multiple range test.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>H/apple</th>
<th>BB/apple</th>
<th>PP/apple</th>
<th>H/carrot</th>
<th>BB/carrot</th>
<th>PP/carrot</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>144.7ᵇ</td>
<td>100.3ᵇ</td>
<td>11.9ᵇ</td>
<td>11.3ᵇ</td>
<td>56.0ᵇ</td>
<td>50.3ᵇ</td>
</tr>
<tr>
<td>20</td>
<td>276.7ᵃ</td>
<td>131.3ᵇ</td>
<td>24.0ᵇ</td>
<td>39.3ᶜ</td>
<td>135.0ᵇ</td>
<td>79.7ᶜ</td>
</tr>
</tbody>
</table>

contrast, more rapid K⁺ leakage was caused by that obtained from infected carrots and the leakage with this isolate tended to be less. There is no evidence from these results for any host tissue selectivity and they are in agreement with the generally accepted view that isolates of *B. cinerea* do not exhibit host specificity.

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REFERENCES


