SHORT COMMUNICATION

Natural Inhibitors of Fungal Polygalacturonases in Infected Fruit Tissues

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(Received 14 July 1980; revised 27 October 1980)

Isoelectric focusing of extracts of plum and peach tissue infected with *Monilinia* spp. revealed the presence of polygalacturonase even though little or no activity was detectable in crude extracts prior to fractionation. Mixing experiments confirmed the presence of an inhibitor of polygalacturonase which was separable from the enzyme by isoelectric focusing. The inhibitor was macromolecular, polydisperse and heat-labile, and its activity was destroyed by trypsin. Similar inhibitors were found in other host–pathogen combinations.

INTRODUCTION

The inactivation of fungal extracellular pectolytic enzymes by oxidized polyphenolics formed on infection or injury of fruit tissue has been suggested as a natural wound defence mechanism (Byrde *et al.*, 1960). The presence of various inhibitors formed on infection (Albersheim & Anderson, 1971; Fisher *et al.*, 1973) may therefore be significant in host–pathogen relations.

During a study of pectolytic enzymes secreted by a number of plant pathogenic fungi in host tissues, little or no endopolygalacturonase [PG; poly(1,4-a-D-galacturonide) glycanohydrolase; EC 3.2.1.15] was detected in certain of the initial extracts, including those of *Monilinia fructigena* in plum fruits. Similar observations on the apparent absence of PG were made by Skare *et al.* (1975) in homogenates of cucumber infected with *Cladosporium cucumerinum* and by Tronsmo & Tronsmo (1977) in extracts of apple fruit infected by *Botrytis cinerea*.

This paper describes preliminary evidence for PG inhibitors in infected fruit tissue which may explain the low recoveries of such enzymes frequently reported (Byrde & Archer, 1977).

METHODS

Tissue extraction. Fruit tissues, including those of apple (*Malus sylvestris*), peach (*Prunus persica*) and plum (*Prunus domestica*), infected with *Monilinia fructigena* (Aderh. & Ruhl.) Honey, *Monilinia laxa* (Aderh. & Ruhl.) Honey, *Monilinia laxa* f. sp. *mali* (Wormald) Harrison, *Pezicula malicorticis* (Jacks) Nannf., *Botrytis cinerea* Fr., *Nectria galligena* Bres. or *Penicillium* sp. were used as sources for extraction. Infected tissues of marrow (*Cucurbita pepo*) and grape (*Vitis vinifera*) were also examined. Material was chosen for extraction while the infection was still progressing. Cultures of *Rhizopus stolonifer*, *R. sexualis* and *Mucor mucedo* for pectolytic enzyme production (Archer, 1979) were made from isolates kindly supplied by Dr S. A. Archer. *Phytophthora infestans* PG was obtained from a focused extract of naturally infected potato leaf.

Samples of rotted and healthy tissue were excised, frozen and lyophilized. Duplicate samples of each host–pathogen combination were prepared. Powdered dried tissue (10 g) was extracted with 100 ml 0-05 M-Tris/HCl buffer plus 0-1 M-KCl at pH 7-6 with cysteine hydrochloride (10 mg l⁻¹) to decrease oxidation. The extract was adjusted with 1 M-NaOH to pH 6-5 after mixing and left for 1 h at 4 °C prior to filtering through Whatman no. 41 paper. The extract was then dialysed against distilled water for 24 h at 4 °C. The dialysed extracts were electrofocused on an LKB 110 column (Laborda *et al.*, 1973) using LKB Ampholines of pH range 0022-1287/81/0000-9432 $02.00 © 1981 SGM
3-5 to 10.0 for 48 h. The column was eluted by a peristaltic pump at approximately 120 ml h⁻¹ into 3 ml fractions, which were then assayed for pH, PG activity and for the presence of an inhibitor of PG activity.

**Enzyme assays.** Polygalacturonase was estimated by the cup plate assay method of Dingle et al. (1953), using a sodium pectate gel. Activities are expressed relative to an aqueous solution (1 mg ml⁻¹) of Pectinol 10 M (Rohm & Haas) defined as having 100 units of PG activity ml⁻¹. Results with this method have been shown to give good correlation with those using a viscometric assay (Fielding & Byrde, 1969), providing that dilutions are made of activities greater than 1000 units. Activity peaks at pH values above 9-0 were checked viscometrically as certain extracts were found to give opaque rings in the gel at about this pH.

Pectin lyase (poly(methoxygalacturonide) lyase; EC 4.2.2.10), pectin esterase (pectin pectylhydrolase; EC 3.1.1.11) and α-L-arabinofuranosidase (α-L-arabinofuranoside arabinofuranohydrolase; EC 3.2.1.55) were assayed as described by Byrde & Fielding (1968) and Willetts et al. (1977).

Protease was determined by a cup plate assay technique using a 1% (w/v) gelatin, 2% (w/v) agar gel in 0.1 M-sodium acetate solution at either pH 3-5 or 5-5. After incubation overnight at 25 °C, the gels were developed using a solution of mercuric chloride (15%, w/v, in 2.5 M-HCl).

**Inhibitor assay.** A polypectate gel similar to that for the PG assay was used, but a mixture of the enzyme and the potential inhibitor (1:2, v/v) was added to each well. The PG used for the assay was generally the active peak resolved in the particular run being tested. When this peak was not sufficiently active, a partially purified PG isoenzyme of pl 9-7 from the focused fractions of a culture filtrate of Monilinia fructigena was used. The focused fractions of the two isoenzymes of pl 4-6 and 5-6 were also used in certain tests.

Preparation of inhibitor. Lyophilized plum tissue (50 g) rotted by M. fructigena was extracted as described for the preparation of enzyme samples except that the dialysed filtrate was adjusted to pH 8-5 with 1 M-NaOH and concentrated to approximately 50 ml by freeze-drying. After a further 24 h dialysis the precipitate was removed by centrifugation. The filtrate was then applied to a column (3 cm diam., 100 ml bed vol.) of DEAE-Sephadex buffered at pH 9-0 with Tris/HCl buffer (0.05 M), calibrated with four marker proteins. The filtrate was then applied to a column (3 cm diam., 100 ml bed vol.) of DEAE-Sephadex buffered at pH 9-0 with Tris/HCl buffer (0.05 M). After an initial washing with the equilibrating buffer plus 0.1 M-KCl (100 ml) the elution was continued with 0.1 M-sodium acetate/acetate buffer at pH 4-8 with stepwise increases in concentration of KCl from 0-1 M to 0-5 M, 1 M and 2 M. The eluted fractions (10 ml) were assayed for inhibitor using a PG preparation of nominal pl 12-0 from rotted plum tissue (adjusted to pH 8-5 after isoelectric focusing). The fractions found to be inhibitory were combined, dialysed and concentrated by freeze-drying.

The inhibitory material was then dissolved in 10 ml 25% saturated (NH₄)₂SO₄ and applied to a small column (1.2 cm diam., bed vol. 20 ml) of Octyl-Sepharose. Elution from the column was stepwise with increasing amounts of ethylene glycol and decreasing amounts of (NH₄)₂SO₄. Inhibitory fractions were dialysed to remove the eluant using dialysis tubing previously boiled in 0-01% EDTA for 1 h and thoroughly washed; this treatment prevented loss of inhibitor by adsorption on the Visking membrane (R. S. Vickery, personal communication). The volume of the dialysis residue was reduced to 10 ml by freeze-drying and the A₂₈₀ was recorded.

**Chemical analyses.** Protein was determined either by absorption at 280 nm or by the method of Lowry with bovine serum albumin as standard. The phenol–sulphuric acid method of Dubois et al. (1956) was used for the estimation of total carbohydrate.

**Molecular weight determinations.** These were carried out using a column of Biogel P-100 (3 cm diam., bed vol. 300 ml) in Tris/HCl buffer (0-05 M, pH 7-6) plus 0-1 M-KCl, calibrated with four marker proteins — cytochrome c, trypsin inhibitor, ovalbumin and bovine serum albumin (monomer) (Andrews, 1964).

**RESULTS**

The pl values of the isoenzymes distinguished in extracts of the various infected host tissues are given in Table 1. As with in vitro cultures of Monilinia spp. (Willetts et al., 1977), the major peak of PG activity in most of the host–pathogen combinations examined occurred at pl values between 9 and 10.

The Penicillium sp. infection on apple was notably different because almost all the PG was of pl 7-9, and only a small peak of activity was detected, on one sample, of pl 9-6. Tissue extracts prepared from peach and plum fruits rotted by M. laxa or M. fructigena contained little or no PG activity. When subjected to isoelectric focusing, these extracts showed a peak of PG activity at about pH 10 or above. The PG peak from each extract was used to test for the presence of an inhibitor in those remaining fractions that showed very little or no PG activity. Inhibitory activity was most marked in the plum and peach extracts, but for all the other host–pathogen combinations examined, with the exception of the Penicillium sp. rot of apple, some inhibitory material was evident. No such inhibitors were detected in focused
Table 1. Polygalacturonase isoenzymes and associated inhibitors detected in vivo

<table>
<thead>
<tr>
<th>Host</th>
<th>Pathogen</th>
<th>pI</th>
<th>Relative activity*</th>
<th>% inhibition</th>
<th>Minor PGs pl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apple</td>
<td><em>Monilinia fructigena</em></td>
<td>10-0</td>
<td>+</td>
<td>4.9-5.2</td>
<td>96</td>
</tr>
<tr>
<td>Apple</td>
<td><em>Monilinia laxa</em></td>
<td>10-1</td>
<td>+</td>
<td>5.9-6.3</td>
<td>90</td>
</tr>
<tr>
<td>Apple</td>
<td><em>Nectria galligena</em></td>
<td>10-0</td>
<td>+</td>
<td>3.0-7.0</td>
<td>90</td>
</tr>
<tr>
<td>Apple</td>
<td><em>Pezicula malarticis</em></td>
<td>9-6</td>
<td>+</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Apple</td>
<td><em>Botrytis cinerea</em></td>
<td>8-4</td>
<td>+</td>
<td>4.3</td>
<td>98</td>
</tr>
<tr>
<td>Apple</td>
<td>(<em>isolate 1)</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apple</td>
<td><em>Botrytis cinerea</em></td>
<td>9-6</td>
<td>+</td>
<td>7.1</td>
<td>98</td>
</tr>
<tr>
<td>Apple</td>
<td>(<em>isolate 2)</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apple</td>
<td><em>Penicillium sp.</em></td>
<td>7-9</td>
<td>+++</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Pear</td>
<td><em>Monilinia fructigena</em></td>
<td>9-8</td>
<td>+</td>
<td>4.3-6.3</td>
<td>99</td>
</tr>
<tr>
<td>Peach</td>
<td><em>Monilinia fructigena</em></td>
<td>9-6</td>
<td>+</td>
<td>4.4-6.0</td>
<td>98</td>
</tr>
<tr>
<td>Plum</td>
<td><em>Monilinia fructigena</em></td>
<td>10-1</td>
<td>+</td>
<td>5.2-5.6</td>
<td>100</td>
</tr>
<tr>
<td>Plum</td>
<td><em>Monilinia laxa</em></td>
<td>12-0†</td>
<td>+</td>
<td>3.8-8.8</td>
<td>92</td>
</tr>
<tr>
<td>Plum</td>
<td><em>Monilinia fructigena</em></td>
<td>10-4†</td>
<td>+++</td>
<td>3.0-4.5</td>
<td>99</td>
</tr>
<tr>
<td>Marrow</td>
<td><em>Botrytis cinerea</em></td>
<td>9-7</td>
<td>+</td>
<td>4.5-5.5</td>
<td>60</td>
</tr>
<tr>
<td>Grape</td>
<td><em>Botrytis cinerea</em></td>
<td>9-7</td>
<td>+</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* Activity (units ml⁻¹): +, <1000; +++, 1000-5000; +++, 5000-10000; ++++, >10000.
† A nominal value is given as the actual value is associated with the electrode solution.

extracts of any healthy tissues. The various host-pathogen combinations examined and the pH range on the focusing gradient within which the inhibitor accumulated are listed in Table 1.

Although the test for the inhibitor was generally made with the PG of pI 9 to 10 from the in vivo extract under test, other PG isoenzymes were examined for comparison. The inhibitor isolated from the apple tissue infected with *M. laxa* f. sp. *mali* was effective against the PGs of pI 5.6 and 9.7 from a culture filtrate of *M. fructigena*, but with a different pattern and level of response. The inhibitor present in *M. fructigena*-infected apple tissue was effective against the PG of pI 9.7 but not against the PG of pI 5.6. The inhibitor isolated from marrow tissue infected with *Botrytis cinerea* inhibited the PG of pI 5.6 from *M. fructigena* but not those of pI 4-6 or 9-7, although the *B. cinerea* PG of pI 9-7 from the marrow infection was inhibited.

Properties of the isolated inhibitor

The inhibitory activity in an extract of plum tissue rotted by *M. fructigena* was retained by DEAE-Sephadex and eluted as a broad peak. Similar results were obtained with Octyl-Sepharose.

The partially purified inhibitor, which was colourless, was found to have a molecular weight of approximately 15 000 when measured using a calibrated column of Biogel P-100. Attempts at molecular weight determination by using Sephadex G-100 resulted in the loss of the inhibitor by adsorption. Efforts to elute the inhibitor with sucrose (1 M), α-methyl glucoside (1 M), Triton X-100 (0.2%, w/v), or by raising the pH, were unsuccessful.

A temperature inactivation curve for a 20 min exposure showed loss of activity from 40 to 70 °C with reduced loss around 80 °C, but complete inactivation at 90 and 100 °C. Heating to 100 °C for only 5 min resulted in a 50% loss of activity.

Tests with the inhibitor against a number of PG preparations isolated from other organisms showed no inhibitory activity; these included *Rhizopus sexualis*, *R. stolonifer*, *Mucor mucedo*, *Phytophthora infestans* and *Penicillium* sp. The inhibitor was ineffective...
against a protease prepared from apple tissue infected with *Monilinia fructigena* (Hislop *et al.*, 1979) and also against pectin esterase, pectin lyase and α-L-arabinofuranosidase from *M. fructigena*.

The amounts of protein (Lowry or $A_{280}$) and polysaccharide in the inhibitor were at the limits of detection (5 μg and 10 μg, respectively) in a 2 mg sample. However, incubation with trypsin (1 mg ml$^{-1}$ in 0.1 M-Tris/HCl buffer, pH 7.5) for 18 h inactivated the inhibitor.

**DISCUSSION**

The PG profiles obtained following isoelectric focusing of extracts of fruit infections seem to reflect the degree of specialization of the pathogens. Thus, the specialized pathogens *Monilinia fructigena, M. laxa f. sp. mali, Nectria galligena* and *Pezicula malicorticis* showed a peak with PI 9.5 or more. However, extracts of apple tissue infected by the unspecialized and opportunistic *Penicillium* sp. showed a main peak at PI 7.9 with or without a lesser peak at PI 9.6. Three different strains of the ubiquitous wound pathogen *B. cinerea* gave two different patterns on apple, with the main peak at either PI 9.6 or 8.4, suggesting the existence of strain differences generally typical of this species (Grindle, 1979).

Inhibitors were present in all the infected fruit extracts examined with the exception of the *Penicillium* sp. rot of apple, but were not detected in any of the healthy tissue extracts. The infection process thus seems to initiate the formation of inhibitors in certain tissue, in all instances their presence being concurrent with tissue oxidation. The exceptional non-oxidative infection of *Penicillium* sp., possibly due to the presence of a phenolase inhibitor, as postulated by Walker (1969) for *P. expansum*, produces no PG inhibitor, thus ensuring the continuation of pectolysis (Cole & Wood, 1961; Byrde & Archer, 1977).

Many of the physical properties of the purified inhibitor are similar to those of an inhibitor studied by Albersheim & Anderson (1971), prepared from the red kidney bean, *Phaseolus vulgaris*. There was, for example, evidence of specificity of action between the inhibitor and the PGs from different fungi as well as differences depending on the particular isoenzyme. The mean molecular weight was considerably lower: 15 000, compared with the 50 000 recorded by Albersheim & Anderson (1971). Although the chemical nature of the inhibitor has not been established, a protein component is suggested by its inactivation by trypsin. The heterogeneity of the inhibitor was demonstrated by its failure to form a sharp peak on isoelectric focusing, and also by its slow elution from Octyl-Sepharose and Biogel P-300, neither gel giving good resolution.

Although the origin of the inhibitors is unknown, the varying ranges and patterns of their separation on the pH gradient in focused extracts of different tissues suggest a host origin. There is at present no evidence concerning whether they might exist in healthy tissue in a latent form which is liberated or activated by the pathogen, or whether they are synthesized *de novo* following infection.

The author is grateful to Dr R. J. W. Byrde for his constant and helpful criticism during the course of this work and in the preparation of the manuscript.

**REFERENCES**


Byrde, R. J. W. & Fielding, A. H. (1968). Pectin methyl-trans-eliminase as the maceration factor of *Sclerotinia fructigena* and its significance in brown


