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SHORT COMMUNICATION

PECTIC ENZYME ACTIVITY FROM *PHYTOPHTHORA INFESTANS*

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Abstract—Filtrates from cultures of *Phytophthora infestans* and inoculated potato tuber tissue contain an endo-polygalacturonase which is separable from galactanase by gel filtration on Sephadex G 100. Pectic lyase and macerating activities were not detected.

INTRODUCTION

IT IS commonly believed that pectic enzymes are implicated in the infection of plants by fungi and bacteria. Many plant pathogenic fungi have been found to produce these enzymes and this ability may be an important part of their pathogenic capabilities.[1, 2] There are only two reports of pectic enzyme production by *Phytophthora infestans*. Grossman[3] detected pectinmethylesterase (PME) activity in filtrates from cultures grown on a specially defined medium. He also indicated that the filtrates reduced the viscosity of pectic and pectinic acid substrates but there was no increase of reducing groups and no detectable galacturonic acid was observed. An endo-polygalacturonase (endo-PG), however, was reported present on these somewhat superficial grounds. Clark[4, 5] confirmed PME production but could not detect exo- or endo-PG or polymethylgalacturonase from filtrates of cultures grown on a wide variety of media. The only other reported extracellular enzyme activity from *P. infestans* is an enzyme which catalyses the liberation of galactose from potato and lupin pectin.[6] There is evidence that the enzyme cleaved chains of β-1,4-linked galactose residues in a random fashion.[7]

The present study was undertaken to examine the extracellular pectic enzyme activity *in vitro* and *in vivo*.

RESULTS AND DISCUSSION

Seven-day-old culture filtrates of *Phytophthora infestans* were tested for pectinase and galactanase activity (Table 1). Chromatography of the reaction products after 24 hr incubation of enzyme and citrus pectin, sodium polypectate (NaPP), polygalacturonic acid (PGA) and apple pectin revealed galacturonic acid, galactose (except in the case of PGA) and a series of spots between the galacturonic acid spot and the base line thought to be oligomers of

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[2] R. K. S. WOOD, *Physiological Plant Pathology*, Blackwell, Oxford (1967).
[3] F. GROSSMAN, *Naturwiss.* **50**, 721 (1963).
[4] D. D. CLARKE, Ph.D. Thesis, University of Hull (1964).
[5] D. D. CLARKE, *Nature* **211**, 649 (1966).
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[7] M. KNEE, Ph.D. Thesis, University of Hull (1968).

galacturonic acid by nature of their orange-brown colour with the aniline reagent.[8] The regularity of the successive $R_{\text{galacturonic acid}}$ values indicated increasing molecular complexity. The $\log R_{\text{galacturonic acid}}$ of these spots when plotted against the probable number of residues gave a straight line and the ratios of movement obtained by each successive spot conformed very well. The series disclosed by this chromatographic data may with some justification be identified as mono-, di-, tri- and tetra-galacturonic acids.[9] This indicates the presence of an endo-polygalacturonase. Galactose and a slower running spot thought to be an oligomer of galactose by virtue of the grey colour produced with the aniline reagent, were identified in the reaction mixture containing potato pectin.

TABLE 1. ACTIVITY AGAINST VARIOUS SUBSTRATES OF CRUDE ENZYME PREPARATION FROM CULTURE FILTRATES OF *Phytophthora infestans*

Substrate	μ-moles reducing groups liberated	
	90 min	24 hr
Citrus pectin N.F.	0·20	1·20
Apple pectin	0·53	1·72
NaPP	0·22	2·94
PGA	—	1·39
Potato pectin	0·30	2·94

Pectic lyase activity was not detected in 7-day-old culture filtrate at pH 3–9 in the presence or absence of 10^{-3} M $CaCl_2$.

Separation of polygalacturonase from galactanase was achieved by gel-filtration on Sephadex G–100 (Fig. 1). A single polygalacturonase peak was obtained. The minor galactanase peak of fraction 18 may be accounted for by the activity of PG on the potato pectin which, although largely galactan, contains some galacturonic acid residues.[7] Chromatography of the reaction products of the PG fractions revealed oligomers of galacturonic acid from both pectin N.F. and polygalacturonic acid substrates. Activity on PGA was much greater than on pectin, suggesting that the polygalacturonase to be an endo-polygalacturonase.[1] Chromatography of the reaction mixtures containing galactanase and potato pectin revealed a strong galactose spot with a series of three spots between it and the base-line. The $\log R_{\text{galactose}}$ /number of residues suggests these to be the dimer, trimer and tetramer of galactose.

The remainder of fractions 12–20 and 22–23 were combined and concentrated by ultrafiltration. The concentrated PG fraction when tested on PGA showed a 288-fold purification from the crude culture filtrate. The combined fractions were tested for macerating activity. No maceration of potato tuber discs occurred over 24 hr using either the polygalacturonase or the galactanase preparation.

Evidence for galactanase activity in infected potato tuber discs has been presented by Knee.[7] Polygalacturonase has now been found to be present in inoculated tuber tissue. Extracts of inoculated tissue were tested on PGA at pH 4·0. After 24 hr incubation the reducing groups liberated were assayed and compared with uninoculated tissue extracts (Table 2). A marked increase in reducing groups occurred after incubation of the substrate

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[9] M. A. JERMYN and R. G. TOMKINS, *Biochem. J.* **47**, 437 (1950).

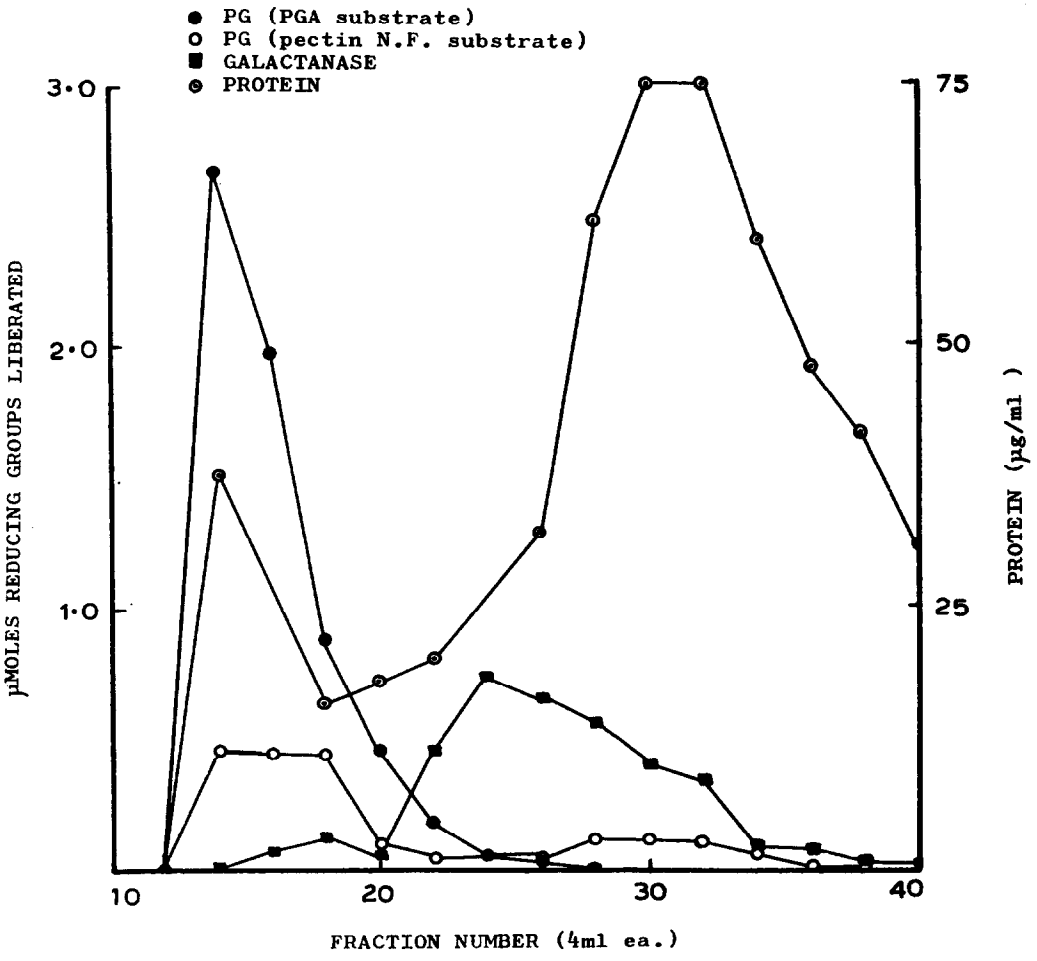

FIG. 1. THE GEL FILTRATION PATTERNS ON SEPHADEX G 100 OF POLYGALACTURONASE ON PGA AND PECTIN N.F. SUBSTRATES AND GALACTANASE IN THE 80% $(NH_4)_2SO_4$ FRACTION OF A 7-day-old CULTURE FILTRATE OF *P. infestans*.

with the preparation from inoculated tissue. Paper chromatographic analysis of the reaction mixture revealed a trace of monomeric galacturonic acid and a series of its oligomers, indicating endo-polygalacturonase activity.

TABLE 2. POLYGALACTURONIC ACID DEGRADING ACTIVITY OF ENZYME PREPARATIONS FROM TUBER DISCS

Tissue	μ-moles galacturonic acid equivalent formed in 24 hr
Uninoculated	0·07
Inoculated	1·00

It has been suggested[7] that galactanase is involved in the attack or penetration of potato tissue by *P. infestans* and it is likely that polygalacturonase would assist in this process. Further investigations into the role of extracellular enzymes in infection are being continued.

EXPERIMENTAL

Chemicals

Citrus pectin N.F., sodium polypectate (NaPP) and polygalacturonic acid (PGA) was supplied by S. & S. Services Ltd., London. Apple pectin was obtained from Koch-Light Laboratories Ltd. Before use, all pectin samples were stirred into 60% (v/v) ethanol made 0·1 N with HCl, followed by thorough washing on a Büchner funnel with 95% (v/v) ethanol. The washed pectins were dried at room temperature. Potato pectin was prepared from King Edward potato tubers according to the procedure of Knee and Friend.[6]

Source and Culture of Fungus

A race 4 isolate of *Phytophthora infestans* (Mont.) de Bary from the culture collection, University of Hull, was used throughout. Mycelial discs from 7-day-old cultures on pea agar[4] were placed in medical flats containing 25 ml of "Birds-Eye" french bean liquid[10] as well as 2 g/l of casamino acids.[6]

Enzyme Preparation

After 7 days growth at 23° the cultures were harvested by filtration through several layers of muslin and centrifuged at 10,000 *g* for 10 min to remove any cells. The crude culture filtrate was then dialysed against 5 mM phosphate buffer (pH 7·0) for 4 hr at 4° and used directly. Alternatively pooled culture filtrates were brought to 80 per cent saturation with $(NH_4)_2SO_4$ at room temperature. The resultant precipitate was collected by centrifugation, resuspended in a minimal volume of 5 mM buffer (pH 7·0). This preparation was stable in storage at −20° for a period of weeks.

Enzyme from inoculated King Edward potato tuber tissue was prepared according to the method of Knee.[7]

Enzyme Assays

Boiled culture filtrate was used as a control in all assays. Pectic hydrolase activity was assessed by estimating the release of reducing groups in reaction mixtures by Nelson's[11] modification of the Somogyi method.[12] Reaction mixtures contained 1 ml substrate (5 mg/ml solutions of pectin N.F., apple pectin, sodium polypectate or polygalacturonic acid), 1 ml buffer and 1 ml enzyme preparation.

Pectic lyase activity was determined spectrophotometrically by following the increase in absorption of reaction mixtures at 230 nm with polygalacturonate substrate and 235 nm with pectin N.F. substrate.[13] The reaction mixtures were as described above. Lyase activity was confirmed using a modification (Bateman, personal communication) of the thiobarbituric acid assay of Neükom.[14]

Galactanase activity was assayed by the reducing group procedure given previously. Reducing sugar released in reaction mixtures containing 1 ml potato pectin solution (5 mg/ml), 1 ml citrate/phosphate buffer (pH 4·0) and 1 ml enzyme preparation, was estimated in terms of galactose.

All enzyme assays were performed at 25° in the presence of toluene.

Macerating activity was measured essentially by the method of Brown.[15] Cylinders of turgid tissue were cut with a number 5 cork-borer from the medulla of a potato tuber. Discs 0·3 mm thick were cut from the cylinder and infiltrated with water under an air pump. Activity was estimated by placing four discs in the test solution consisting of 1 ml enzyme preparation and 1 ml buffer, and testing at intervals by gentle pulling between forceps.

Identification of Products

The products of the enzyme reactions were subjected to chromatography on Whatman No. 1 paper with galacturonic acid, galactose, glucose and arabinose standards, using *n*-BuOH–HOAc–H_2O (6:1:2, by vol.).[8] Papers were developed with the $AgNO_3$ reagent.[9] Reaction products were also identified by TLC on Cellex MX (Calbiochem). The plates were irrigated twice with pyridine–EtOAc–HOAc–H_2O (5:5:1:3, by vol.).[16] Reaction products were detected with the aniline reagent.[8]

Gel Filtration Chromatography

Pectinase and galactanase were separated by gel filtration of the 80% $(NH_4)_2SO_4$ fraction on a 2·5 × 37 cm column of Sephadex G 100. Filtration was carried out at 4° in the presence of 0·05 M citrate/phosphate buffer, pH 4·0, and 4-ml fractions collected.

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