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COMPARISON OF THE PROPERTIES OF THE PECTIN TRANSELIMINASES OF *PENICILLIUM DIGITATUM* AND *PENICILLIUM ITALICUM*

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Abstract—The production and purification of pectin transeliminase of *Penicillium italicum* is described and certain properties of this enzyme are compared with the pectin transeliminase of *P. digitatum*. Evidence is presented for the production of pectin transeliminase *in vivo* in oranges rotted by these fungi. The contribution of pectin transeliminase in the decay syndromes produced by these Penicillia is indicated.

INTRODUCTION

IN A PREVIOUS publication[1] it was shown that the ability of *Penicillium digitatum* to macerate orange-rind tissue was due to the production by the fungus of the enzyme pectin transeliminase (PTE). When purified until the preparation showed only one protein band on disc electrophoresis and no other enzyme activity could be detected, the PTE still proved capable of macerating orange-rind tissue. This observation is in agreement with the work of McClendon[2] and with the views of Bateman and Millar,[3] indicating that either endo-polygalacturonase (PG) or endo-PTE may be implicated in maceration. Further evidence for the ability of PTE to cause maceration of plant tissues has been found by Byrde and Fielding[4] using *Sclerotinia fructigena.*

When filtrates from *Sclerotinia* cultures were fractionated by dextran gel filtration or by ion-exchange chromatography on substituted cellulose, macerating activity was found to be associated with the PTE activity and not with the endo-PG or α-L-arabanofuranosidase activity. Byrde and Fielding however, questioned the role of PTE in the pathogenesis of *Sclerotinia* as they were unable to detect any significant quantities of this enzyme *in vivo.*

P. italicum like *P. digitatum* causes post harvest decay of citrus fruit. The infections of fruit caused by these fungi may be distinguished by the olive or dull yellow-green spores and smooth velvety colonies of *P. digitatum*, causing an initial softening of the fruit which in conditions of moderate humidity eventually shrinks and dries up. Infection by *P. italicum*, which gives rise to restricted colonies with blue-green spores, reduces the fruit to a soft pulpy condition. The colonies of *P. italicum* are further distinguished by a more granular texture and the formation of prostrate coremia at the colony margin.

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[1] D. A. BUSH and R. C. CODNER, *Phytochem.* **7**, 863 (1968).
[2] J. H. MCCLENDON, *Am. J. Botany* **51**, 628 (1964).
[3] D. F. BATEMAN and R. L. MILLAR, *Ann. Rev. Phytopath.* **4**, 119 (1966).
[4] R. J. W. BYRDE and A. H. FIELDING, *J. gen. Microbiol.* **52**, 287 (1968).

In the present work PTE from *P. italicum* has been isolated and purified in the same way as was the PTE of *P. digitatum*. The purified PTE of *P. italicum* has also been shown to cause maceration of orange tissue. Certain properties of the purified PTE preparations from the two Penicillia have been compared and their *in vivo* production in oranges has been investigated.

RESULTS

1. *Purification of PTE from* Penicillium italicum

Preliminary work indicated that the highest activities of both PTE and macerating activity were obtained when *P. italicum* was grown on moistened bran. 4 l. of *P. italicum* bran culture extract were prepared as described below, freeze dried and reconstituted to 1 l. with distilled water. The concentrated bran extract was subjected to fractional precipitation with ammonium sulphate and the fractions were redissolved in 100 ml distilled water (Table 1). The solution of the precipitate formed at 0·5 ammonium sulphate saturation was subjected to a second

TABLE 1. FIRST AMMONIUM SULPHATE FRACTIONATION OF *Penicillium italicum* FROM CULTURE EXTRACT

Fraction	Pectin transeliminase		Arabanase		Polygalacturonase		Pectin methyl esterase (presence in plate assay)	Maceration
	Activity (0·01 absorptivity/ml/min)	Specific activity (units activity/mg protein)	Activity (μg arabinose liberated/min)	Specific activity (units activity/mg protein)	Activity (μmoles reducing group/min)	Specific activity (units of activity/mg protein)		
Bran extract	71	15·0	26·7	6·3	0·32	0·15	+	+
Concentrated bran extract $(NH_4)_2SO_4$ saturation	154	16·9	61·7	7·6	0·64	0·16	+	++
0·2	120	6·6	0	—	0	—	—	++
0·4	245	49·7	126·7	25·7	0	—	—	++++
0·5	760	253·3	126·7	42·2	0	—	—	+++*
0·6	239	52·5	20·0	4·0	0	—	—	+++
0·8	22	1·7	90·0	6·9	1·96	0·30	+	+
1·0	2	0·3	86·7	14·4	1·09	0·36	+	±
Residue (material not precipitated by saturated $(NH_4)_2SO_4$)	0	—	16·7	4·9	0	—	+	—

* Diluted $\frac{1}{4}$ in maceration assay; otherwise undiluted fractions were used.
Key for maceration as in Table 3.

ammonium sulphate fractionation. The precipitates so formed were then dissolved in 15 ml of distilled water and assayed for enzyme activity with the results shown in Table 2.

The solution of the precipitate formed in the second ammonium sulphate fractionation at 0·5 saturation was passed through a column of Sephadex G25 to remove salts and was then subjected to column chromatography on ECTEOLA cellulose, as previously described for *P. digitatum*.[1] On the ECTEOLA column PTE was eluted with distilled water and was thus separated from arabanase and other inactive protein which were eluted from the column using stepwise increases in sodium chloride concentration as shown in Fig. 1.

The specific activity of the PTE was increased from 15 units/mg protein in the bran culture extract to 2170 units/mg protein in the purest fraction (fraction 5) from the ECTEOLA cellulose column, giving a 145-fold overall increase in specific activity. Similarly, the specific activity of the arabanase was increased from 6·3 units/mg protein in the bran filtrate to 697·0 units/mg protein in fraction 16 from the ECTEOLA cellulose column, giving a 110-fold increase in specific activity. As with *P. digitatum*, this procedure did not effect any separation of PTE from macerating activity.

TABLE 2. SECOND AMMONIUM SULPHATE FRACTIONATION OF THE 0·5 SATURATION PRECIPITATE FROM TABLE 1

Fraction	Pectin transeliminase: Activity (0·01 absorptivity units/ml/min)	Pectin transeliminase: Specific activity (units/mg protein)	Arabanase: Activity (μg arabinose/ min)	Arabanase: Specific activity (units activity/ mg protein)	Maceration
0·5 sat. $(NH_4)_2SO_4$ Precipitate from first fractionation $(NH_4)_2SO_4$ saturation	712	250	113	40	++++
0·2	31	67·4	0	—	±
0·4	770	315·6	477	196	++++
0·5	2186	888·6	247	100	++*
0·6	358	303·4	8	7	+++
0·8	40	26·1	8	5	+
1·0	18	24·0	4	5	±
Residue (material not precipitated by saturated $(NH_4)_2SO_4$)	0	—	10	—	—

* $\frac{1}{10}$ dilution, otherwise undiluted fractions.
Key for maceration as in Table 3.

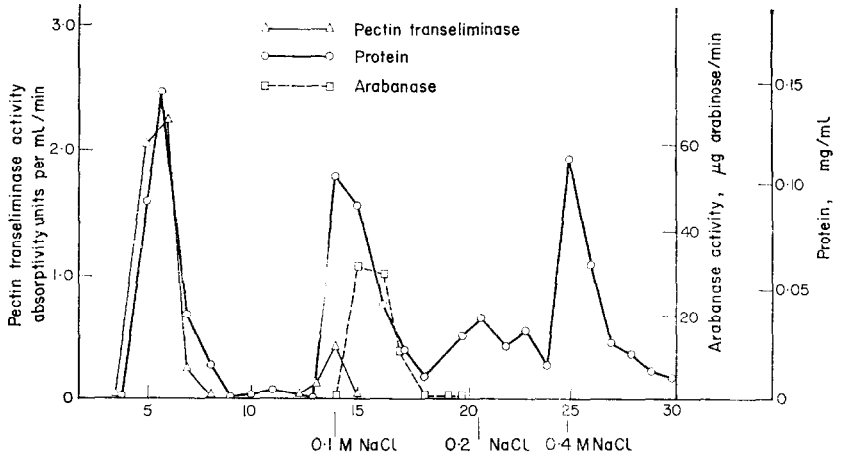

FIG. 1. SEPARATION OF THE ARABANASE AND PECTIN TRANSELIMINASE OF *P. italicum* BY COLUMN CHROMATOGRAPHY ON ECTEOLA CELLULOSE.

2. *Comparison of the Properties of the Purified Pectin transeliminase of* Penicillium digitatum *and* Penicillium italicum

Electrophoretic homogeneity. The PTE purified from *P. digitatum* culture filtrate was earlier found to be electrophoretically homogenous when run on polyacrylamide gels at pH 9·5.[1] The PTE purified from *P. italicum* bran extract in the present work was therefore examined in the same way and was also found to be electrophoretically homogeneous. Samples

of both purified P.T.E.s were then applied as a mixture to the column. Again only one protein band was detected, giving strong evidence for the similarity of the two enzymes.

Absorption spectra of reaction products. The purified PTE's from *P. digitatum* and *P. italicum* were allowed to react with buffered pectin at pH 5·2 for 15 min. After this time the absorption spectra of the reaction products were examined using a Unicam SP 800 recording spectrophotometer, using a buffered pectin solution as a blank. The absorption maxima for both reaction mixtures appeared at about 239 nm rather than the value of 235 nm reported by Albersheim and Killias.[5] Both Cole[6] and Byrde and Fielding[4] have also reported that the reaction products of PTE had an absorption peak higher than 235 nm.

The effect of substrate concentration on the activity of the PTE of Penicillium digitatum *and* Penicillium italicum. The effect of substrate concentration on enzyme activity varies with different enzyme substrate systems. The Michaelis constant, or the substrate concentration

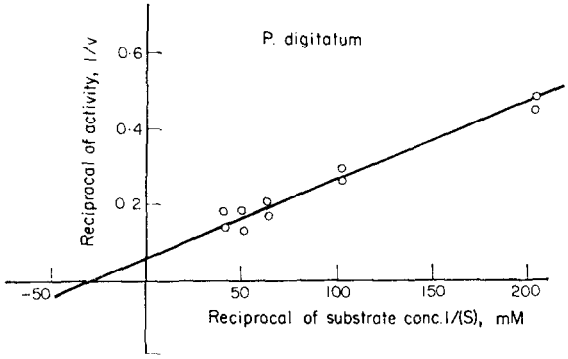

FIG. 2. PLOT OF $1/v$ AGAINST $1/(S)$ FOR *P. digitatum* PTE using method of Lineweaver and Burk.[7]

when half maximum velocity is reached, is a measure of the affinity of an enzyme for its substrate, and can be used to compare different enzyme substrate systems. The usual method[7] of obtaining the Michaelis constant (K_m) is to plot $1/v$ against $1/S$, where v = initial velocity or activity and S = the substrate concentration. K_m is then taken as the reciprocal of the value where the plot bisects the negative part of the abscissa. The substrate concentration is measured in molarities for the purpose of obtaining K_m. For the reaction of PTE involving pectin of unknown molecular weight as substrate it is necessary to taken an arbitrary approximate value for the molecular weight. Molecular weights for pectin have been reported to vary from 20,000 to 200,000. Thus, as the pectin used in the present work was an extracted material, and was likely to have been considerably depolymerized during extraction, it was decided to take the lower value as the approximate molecular weight for the calculation of K_m. The Michaelis constants obtained by this method are not therefore absolute values, but can be used to compare the PTEs from each organism. The reaction of each PTE was thus measured at a variety of substrate concentrations and the results are plotted in Figs. 2 and 3. From these, the apparent K_m value for both enzymes was 0·003 mM.

Effect of pH on PTE activity. Activities of purified PTE samples from both Penicillia were measured over a range of pH values. The optimum pH for the enzymes from both fungi

[5] P. ALBERSHEIM and U. KILLIAS, *Archs Biochem. Biophys.* **97**, 107 (1962).
[6] A. L. J. COLE, Doctoral Thesis, Univ. Lond. (1967).
[7] H. LINEWEAVER and D. BURK, *J. Am. Chem. Soc.* **56**, 658 (1934).

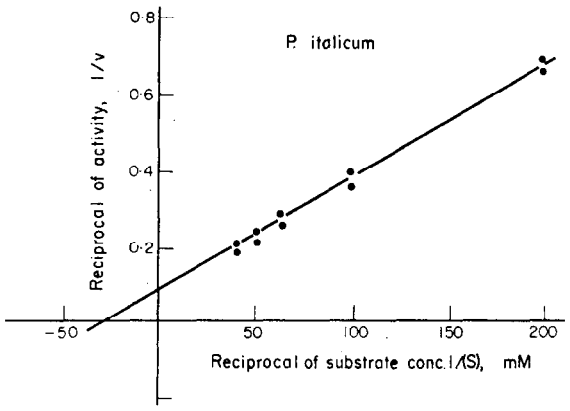

FIG. 3. PLOT OF $1/v$ AGAINST $1/(S)$ FOR *P. italicum* PTE using method of Lineweaver and Burk.[7]

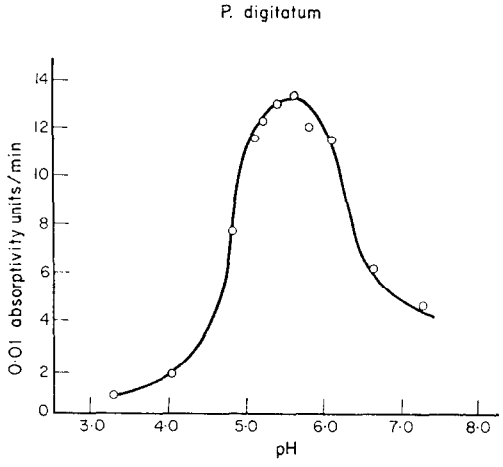

FIG. 4. EFFECT OF pH ON THE ACTIVITY OF *P. digitatum* PTE.

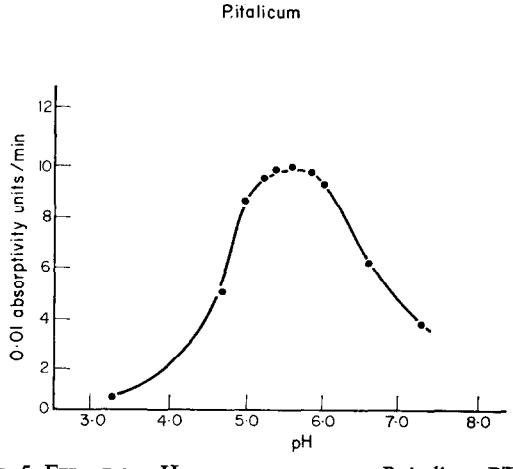

FIG. 5. EFFECT OF pH ON THE ACTIVITY OF *P. italicum* PTE.

was approximately 5·5. This is higher by 0·3 pH units than the value of 5·2 reported by Albersheim and Killias.[5] The effect of pH on PTE activity of the two enzymes is shown in Figs. 4 and 5.

Effect of pH on Viscosity-Reducing Activity of the PTE Preparations

Preliminary experiments had indicated that PTE might be responsible for the reduction in viscosity of pectin solutions, caused by culture filtrates of *P. digitatum* and *P. italicum*. It was subsequently found that the electrophoretically homogeneous PTE preparations from both organisms were able to cause a decrease in viscosity of pectin solutions. To obtain

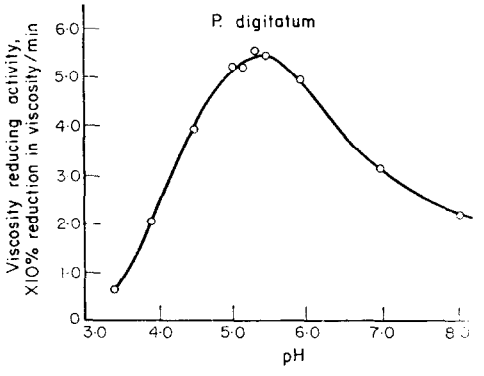

FIG. 6. EFFECT OF pH ON THE VISCOSITY-REDUCING ACTIVITY OF *P. digitatum* PTE.

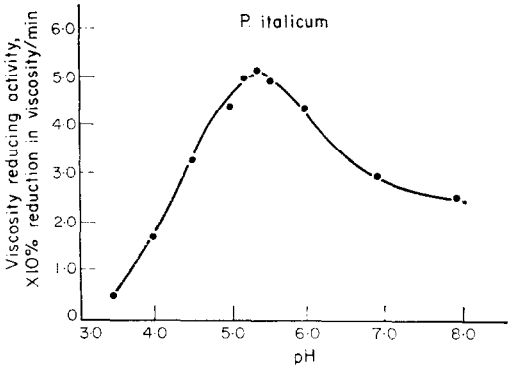

FIG. 7. EFFECT OF pH ON THE VISCOSITY-REDUCING ACTIVITY OF *P. italicum* PTE.

further confirmation that PTE was responsible for the viscosity-reducing activity, the action of the purified PTE preparations in reducing the viscosity of pectin was examined over a range of pH. The activity pH curves obtained in this experiment (Figs. 6 and 7) are of the same form as the activity pH curves found when PTE activity was measured spectrophotometrically. The optimum pH for both types of activity differed by less than half a pH unit. The apparent ability of the PTEs from both organisms to reduce the viscosity of pectin, points very strongly to their being endo-enzymes. The action of an exo-enzyme would be unlikely to produce enough change in the molecular size of pectin to cause the rapid reduction in viscosity observed. This in turn substantiates evidence so far put forward that PTE can

be responsible for maceration of orange tissue, as it is recognized by Bateman and Millar[3] that endo-enzymes are more likely to be responsible for the maceration of plant tissues than exo-enzymes.

Effect of pH on the Maceration of Orange Tissue by the Purified PTE Preparations

Although the macerating activity assay used in the present work is not strictly quantitative, an attempt was made to determine the effect of pH on maceration, within the limits of the assay. 4 ml of 0·1 M McIlvaines citrate phosphate buffer were added to 1 ml of a purified PTE preparations containing approximately 400 units of activity and two orange discs were placed in this solution. Softening of the discs was estimated after 1 and 24 hr at the following pH values: 3·0, 4·0, 5·0, 5·5, 6·0, 7·0, and 8·0. Orange discs were also placed in buffer at the various pH values in order to determine how much softening was caused by the buffer alone. The results are shown in Table 3.

TABLE 3. EFFECT OF pH ON MACERATING ACTIVITY OF PURIFIED PTE PREPARATIONS

	Penicillium digitatum PTE		*Penicillium italicum* PTE		Controls (buffer without enzyme)	
pH	1 hr	24 hr	1 hr	24 hr	1 hr	24 hr
3·0	±	++	—	++	±	++
4·0	+	+++	±	+++	±	+
5·0	+++	++++	++	++++	—	±
5·5	+++	++++	++	++++	—	±
6·0	+++	++++	++	++++	—	—
7·0	±	++	+	++	—	—
8·0	±	+	±	+	—	—

Key: ± very slight softening compared with the control; + softening; ++ extreme softening; +++ partial disintegration; ++++ almost complete disintegration.

In view of the softening which occurred in the controls, at pH 3·0 and 4·0, it is questionable how much softening is due to enzyme activity and how much to pH, in the course of the rotting of fruits by *P. digitatum* and *P. italicum*. The pH obtained when 1 g orange rind is blended in 1 ml water is between 4·0 and 4·5; thus both enzymic and pH maceration could theoretically occur once the integrity of the tissue is destroyed.

Thermal Inactivation of PTE

0·5 ml samples of undiluted, purified PTE were held at temperatures ranging from 50–70° for periods of 5 min. Preliminary experiments had indicated that inactivation occurred in this temperature range. At the end of the immersion period, the 0·5 ml samples were removed from the water bath and diluted to 5 ml with distilled water at room temperature. This ensured that the samples were maintained at the inactivation temperature for exactly 5 min. The samples were then assayed in the normal way. Controls were treated in the same way, except that they were kept at room temperature. The results are shown in Table 4. Percentage reduction in activity was calculated by the formula $R=[(C-A)/C]\times 100$, where $R=\%$ reduction in activity; $C=$activity of control; and $A=$activity of sample after heating.

TABLE 4. THERMAL INACTIVATION OF PTE

Temperature for 5 min (deg)	% Reduction in PTE activity	
	P. digitatum	*P. italicum*
50	2	12
55	3	42
60	39	94
65	96	99
70	100	100
75	100	100

The results show that the PTE preparation from *P. italicum* is inactivated at a lower temperature than is the PTE of *P. digitatum*. This is the only difference which has been observed when comparing the properties of PTE preparations from the two Penicillia. The relative instability of the *P. italicum* PTE is in keeping with the observation that there is a greater loss of *P. italicum* PTE during the purification process. Since the *P. italicum* PTE preparation used had a lower specific activity it is possible that impurities may have had an adverse effect on the thermal stability.

Action of PTE on Pectic Acid

Pectic acid transeliminase (polygalacturonic acid transeliminase, PGTE) was not detected in culture filtrates of either *P. digitatum* or *P. italicum* although a very low level of PGTE activity was detected in fractions from either fungus showing high PTE activity. It seemed likely that the low levels of apparent PGTE were due to traces of methoxyl groups present in the sample of polygalacturonic acid used as substrate. It was found that the very low levels of PGTE activity were only detected within the pH range 4·5–6·0 at which PTE was most active.

Chromatography of the Reaction Products of the Action of PTE on Pectin

Samples of purified PTE from *P. digitatum* and *P. italicum* were mixed with buffered 0·5% pectin solutions and after varying reaction times of up to 24 hr samples of the reaction mixture were examined by TLC.[8] No material was detected in the region of the galacturonic acid marker suggesting that no monomer is produced by these PTE preparations. This indicates that both enzymes were endoenzymes.

Softening of Intact Oranges with Purified PTE

Oranges were injected with 1 ml of purified PTE (about 400 units of activity), from both *P. digitatum* and *P. italicum*. 1 ml portions of boiled enzyme were injected as controls. After 1 hr slight softening was noticed around the sites of injection of the active enzymes, but not around the sites of injection of the boiled enzyme. After 12 hr the softening became more apparent, the softened area turned brown and became sunken. The fruits with these softened areas dried out more quickly than the control fruits. The area of softening remained discrete, and the diameter varied from about 2 to 3 cm. This suggested that the enzyme was absorbed onto the orange tissue fairly quickly and was therefore prevented from diffusing over a wide

[8] YU. S. OVADOV, E. V. EVTUSHENKO, V. E. VASKOVSKY, R. G. OVODOVA and T. F. SOLOVEVA, *J. Chromatog.* **26**, 111 (1967).

area. Alternatively the discrete areas of softening might have been due to the enzyme being inactivated by substances in the rind tissue.

Presence of PTE and other Pectic Enzymes in Orange Tissue Infected with Penicillium digitatum *and* Penicillium italicum

Rind from healthy oranges and from oranges infected with *P. digitatum* or *P. italicum* was extracted with 0·5 M Na_2HPO_4 according to the technique of Cole and Wood.[9] Pectin methyl esterase (PME) was found in extracts of both healthy and infected fruit, whereas PTE was found only in extracts of infected rind. The PTE activity found in infected rind from both Penicillia was about one-twentieth of that found in culture filtrates compared on a w/v basis. Extracts of infected rind also showed the viscosity-reducing activity against pectin which would be expected from the level of PTE present in the infected rind. Low levels of macerating activity were also found only in the extracts of infected rind. Arabanase activity was not found in any of the extracts, although extracts from infected rinds showed a higher free pentose content than did the extract from healthy peel. This pointed to the fact that arabanase may have been active during the invasion of the fruits by the pathogens but had subsequently been denatured possibly by the extraction process or had become strongly bound to the insoluble rind material.

DISCUSSION

It was shown previously that the PTE produced by *Penicillium digitatum* could account for the macerating activity of filtrates of cultures of this fungus. Results obtained with *P. italicum* also show that, by the methods described, it has not been possible to separate macerating activity from PTE. As with *P. digitatum* preparations the purified PTE of *P. italicum*, which still caused maceration, was found to be homogeneous when run on polyacylamide gels at pH 9·5. The electrophoretic mobility of the single band produced by purified *P. italicum* PTE was shown to be identical with that of the *P. digitatum* PTE by co-electrophoresis of a mixture of the purified enzymes from the two fungi. The PTE preparations from the two Penicillia are also similar in a number of other respects. The position and form of the u.v. absorption peak for the reaction products of the two enzymes with pectin are identical. Both had similar affinities for the substrate. The pH optima of PTE preparations from the two Penicillia were identical and the pH activity curves were of the same form when their activity was measured either by increase in absorption at 235 nm of the reaction mixture (Figs. 4 and 5) or by the reduction in viscosity of pectin solutions (Figs. 6 and 7). Both enzymes appear to be endo-enzymes attacking only methylated pectin, as neither produced chromatographically detectable amounts of galacturonic acid monomer in the reaction mixture and neither showed more than a negligible reaction with pectic acid. The two PTEs differed, in the properties examined, only in their stability at elevated temperatures; the *P. italicum* PTE being inactivated at lower temperature than the *P. digitatum* PTE. While it is possible that this result may be due to a structural difference between the two enzymes, it is more likely that the difference is due to the presence of traces of impurities in the *P. italicum* PTE preparation, which had a rather lower specific activity than the *P. digitatum* PTE. The close similarity of the properties of the two enzyme preparations points to their being identical; further evidence for this identity might be obtained by electrophoresis at a range of different pH or by serology.

P. italicum, like *P. digitatum*, produces a PTE which in the absence of other known pectic enzymes is capable of causing maceration of orange rind and purified PTE preparations from

[9] M. Cole and R. K. S. Wood, *Ann. Botany* **25**, 435 (1961).

both fungi cause zones of softening when injected into the rind of intact oranges, clearly indicating the role of PTE in maceration. This observation does not exclude the possibility that other enzymes produced by these fungi may also be involved in maceration. In fact, during the fractionation of the *P. italicum* culture filtrates, evidence was found of some maceration in fractions devoid of PTE activity and it is probable that this was due to polygalacturonase found in these fractions.

It is of interest that the optimum pH for the PTE of both Penicillia is between pH 5·0 and 5·5 while the pH of orange rind blended in distilled water is between pH 4·0 and 4·5. In this pH range PTE from both Penicillia still shows activity, unlike the situation with the PTE components of *Sclerotinia fructigena* where the pH optima for PTE components of the culture filtrates differ widely from the pH of apple tissue.[4]

Wood[10] states that in order to show that an enzyme plays a part in plant disease, it must be shown either to be present and active in infected plant tissue or to have been present and active in such tissue. In the present work, low levels of PTE activity have been demonstrated in orange tissue infected with either *P. digitatum* or *P. italicum*. The presence of macerating factor and pectin viscosity reducing activity have also been detected at low levels in extracts of infected tissue. These results are in contrast with those of Cole[6] who was unable to demonstrate PTE in oranges infected with *P. digitatum*. The detection of enzyme activities in infected tissues is difficult for at least two reasons. Firstly, the enzymes may be inactivated by substances in the tissue; thus Cole[6] found that orange juice contained a thermolabile inhibitor of macerating activity and viscosity-reducing activity. Secondly, enzymes may become adsorbed to the host tissue. If too vigorous methods are used to liberate the adsorbed enzymes this may lead to inactivation of the enzymes. A further difficulty in the demonstration of PTE in infected orange rind is due to the high u.v. absorption at 235 nm of the extract. The sensitivity of the spectrophotometer is then inadequate to detect small increases in absorption. This difficulty was overcome by reading the absorption of reaction mixtures against a boiled tissue extract blank or by using an ammonium sulphate precipitate of the extract rather than the extract itself in the detection of activity. It is suggested that the evidence obtained in the present work satisfies the conditions of Wood[10] and that PTE is clearly implicated in the maceration and rotting of oranges by both *P. digitatum* and *P. italicum* and it is further suggested that PTE plays a significant role in the host–parasite relationships of these fungi with citrus fruits

EXPERIMENTAL

Growth Conditions of Penicillium italicum

P. italicum was obtained from Bath University of Technology culture collection and was maintained on malt extract agar or Czapek Dox agar. Cultures were grown in 1 l. conical flasks, containing 50 g bran and 50 ml water, at 25° in a constant temperature room. The flasks were inoculated with spore suspensions prepared as described[1] for *P. digitatum*. Filtrates were prepared from bran cultures after 72 hr growth by adding water to the bran (100 ml water to 10 g dry bran) and allowing this to stand for an hour. At the end of this time the bran was removed from the liquid by filtering through muslin and the filtrate was centrifuged at 2500 rev/min (2975 × g) for 1 hr. After harvesting, the filtrates were either frozen immediately or freeze dried. Fractionation of culture filtrates, chromatography of reaction mixtures, assays for PTE, arabanase PME and maceration, protein estimations and electrophoresis were all carried out by the methods reported previously.[1]

Reducing Group Assay for Polygalacturonase

The release of reducing groups from pectic acid by culture filtrates was measured by the method of Jansen and McDonnel.[11] Pectic acid was washed in 70% ethanol to remove any traces of reducing sugar impurities

[10] R. K. S. WOOD, *Ann. Rev. Plant Physiol.* **6**, 299 (1960).
[11] E. F. JANSEN and L. R. MCDONNEL, *Archs Biochem.* **8**, 97 (1945).

and was then dissolved in 0·1 M acetate buffer, pH 5·0, to give a 0·5% solution. The pH of the solution was adjusted to 5·0 with 1 N NaOH. To 15 ml of this substrate was added 0·5 ml of enzyme and, after mixing, 5 ml was immediately withdrawn and pipetted into 0·9 ml of M Na_2CO_3 in a glass-stoppered bottle. The enzyme substrate mixture was incubated for 8 min at 30° and a further 5 ml was withdrawn and pipetted into 0·9 ml of M Na_2CO_3 in another glass-stoppered bottle. 5 ml of 0·1 N iodine were then added to each bottle and left for 20 min, at the end of which time 2·0 ml of 2 N H_2SO_4 were added and the mixture was shaken. The contents of each bottle were then titrated against 0·05 N sodium thiosulphate. From the difference in titration values, the number of milliequivalents of iodine used, and consequently the number of millimoles of reducing group liberated could be calculated, having asertained that 1 millieq. of iodine equals 0·530 millimoles of —CHO. The unit of P.G. activity is then defined as the amount of enzyme which causes a liberation of 1 μM of reducing group per min under the conditions described above.

Assay of Viscosity-Reducing Enzymes

Enzymes reducing the viscosity of pectin were assayed by the method of Roboz *et al.*[12] using 0·5% pectin in citrate phosphate buffer, pH 5·2, as a substrate. 0·25 ml of enzyme was added to 7·5 ml of substrate and the time noted. 4 ml of this enzyme substrate mixture were then pipetted into a viscometer immersed in a water bath at 25°, and at recorded time intervals the time taken for the liquid to fall between two points was determined, using a 0·1 sec stop-watch. The viscosity of the substrate and boiled enzyme and the buffer plus boiled enzyme were also measured as controls. The percentage decrease in viscosity was calculated by the following formula:

$$A = \frac{V_0 - V_t}{V_0 - V_s} \times 100$$

where A = percentage decrease in viscosity; V_0 = flowtime in sec of the substrate + heat-inactivated enzyme; V_t = flowtime in sec of the substrate + the enzyme at recorded time intervals; and V_s = flowtime of the buffer + heat-inactivated enzyme.

[12] E. Roboz, R. W. Barratt and E. L. Tatum, *J. Biol. Chem.* **195**, 459 (1952).