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THE NATURE OF MACERATING FACTOR OF *PENICILLIUM DIGITATUM* SACCARDO

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Abstract—The pectic enzymes produced by *Penicillium digitatum*, which invades oranges, have been compared with those produced by *P. notatum* which does not attack oranges. The differences found are discussed in relation to the ability of the culture filtrate of *P. digitatum* to macerate orange-rind tissue. Purification of pectin transeliminase from the culture filtrate of *P. digitatum* is described and evidence is discussed which suggests that it is this enzyme which is responsible for the maceration of orange-rind tissue.

INTRODUCTION

IT IS now widely accepted that macerating enzymes play a large part in the invasion of plant tissues by certain fungal and bacterial pathogens.[1–3] There has, however, been some doubt as to the precise nature of the enzymes causing maceration.

The material forming the middle lamella of plant tissue must be broken down before the cells can become separated, as is the case in macerated tissues. This middle lamellar material consists mainly of pectic substances which, it seems likely, form the substrates for the macerating enzyme systems. It is unlikely that the cellulose components of the cell wall are involved in the maceration process as it appears that the bulk of the cells in the macerated tissue remain intact.

The chemical structures of pectic substances from various plant materials have been studied by many workers but in particular by Hirst and Jones.[4–7] This subject has been comprehensively reviewed by Deuel and Stutz.[8] Basically pectin consists of polymers of galacturonic acid residues joined by 1:4 α-glycosidic links. The carboxyl groups on the galacturonic acid units are methylated to a varying degree, and non-uronide material such as araban and galactan is attached to these chains by covalent linkages.

There are thus a number of different points at which these pectin hetero-polymers may undergo enzymic degradation. These enzyme substrate systems have been reviewed by several workers,[8–10] and can be broadly classified into three groups

1. Pectin methyl esterases (PME) which remove the methyl ester groups from pectin.
2. Enzymes attacking the main galacturonide polymer skeleton, in either the methylated or demethylated state, by either hydrolysis or transelimination. Such attack

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[4] E. L. HIRST and J. K. N. JONES, *J. Chem. Soc.* 496 (1938).
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[6] E. L. HIRST and J. K. N. JONES, *J. Chem. Soc.* 1865 (1939).
[7] E. L. HIRST and J. K. N. JONES, *Advan. Carbohydrate Chem.* II (1946).
[8] H. DEUEL and E. STUTZ, *Adv. Enzymol.* **20**, 341 (1958).
[9] A. L. DEMAIN and H. J. PHAFF, *Wallerstein Lab. Commun.* **20**, 69 (1957).
[10] D. F. BATEMAN and R. L. MILLAR, *Ann. Rev. Phytopath.* **4**, 119 (1966).

may be either on a terminal 1:4 linkage or randomly on within chain 1:4 linkages giving the possibility of exo- and endo-enzymes.

3. Arabanases and galactanases which attack non-uronide substances associated with pectin.

Most of these systems have at some time been implicated in the phenomenon of maceration. Wood[3] quotes suggestions that polygalacturonase (PG) which attacks the main uronide chains of demethylated pectin at the 1:4α-glycosidic links may be associated with the maceration caused by some plant pathogens. Since pectin in plant material is normally considered to be fully methylated, such a macerating system would have to involve a pectin methyl esterase (PME).

Byrde and Fielding[11,12] put forward evidence for the participation in maceration of arabanases which break down the araban polymers associated with pectin, while Fuchs *et al.*[13] have discussed the breakdown of the pectin–araban complex by phytopathogenic fungi. McClendon,[14] in an extensive examination of pectic enzyme production, admits that arabanases may macerate, but suggests that pectin transeliminase (PTE), an enzyme recently described by Albersheim *et al.*,[15] is more likely to be responsible for maceration. This enzyme degrades esterified pectin by a transelimination at some of the 1:4α-glycosidic links, to give residual oligomers with terminal galacturonic acid units having an unsaturation between carbon atoms 4 and 5.

Current evidence[10] suggests that while arabanases may be involved, the pectic enzymes are the major factors responsible for plant tissue maceration. The endo-forms of both hydrolases and transeliminases are capable of macerating plant tissues whereas the exo-types of these enzymes are apparently of little consequence. There is evidence that cellulases do not contribute significantly to the maceration process.[10]

The object of the present work was to determine the nature of the enzyme(s) responsible for the maceration of orange tissue by *Penicillium digitatum*, and to compare the array of pectic enzymes produced by this organism with that produced by *P. notatum*, a related mould which will grow on damaged orange tissue but lacks macerating activity and is unable to invade a whole orange even from an initial lesion.

RESULTS

Chromatographic examination of digests of orange rind by *Penicillium digitatum* culture filtrate showed the presence of arabinose and galacturonic acid, whereas similar digests using *P. notatum* culture filtrate showed only the presence of galacturonic acid. These results suggested that *P. digitatum* produced an arabanase not produced by *P. notatum*, and, also, that both organisms produced an enzyme or enzymes capable of producing galacturonic acid from citrus pectin. This is likely to be an exo-PG which removes terminal galacturonic acid units from pectin after this has been demethylated by PME.

Confirmation that both organisms produced a PME and a PG was obtained using cup-plate assay techniques. Culture filtrates were examined for enzymes of the trans-eliminase type by observing the change in u.v. absorption at 235 nm of a clarified citrus pectin. PTE was detected in *P. digitatum* culture filtrate but not in that of *P. notatum*.

[11] R. J. W. BYRDE and A. H. FIELDING, *Nature* **196**, 1227 (1963).
[12] R. J. W. BYRDE and A. H. FIELDING, *Nature* **205**, 390 (1965).
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[14] J. H. MCCLENDON, *Am. J. Botany* **51**, 628 (1964).
[15] P. ALBERSHEIM, H. NEUKOM and H. DEUEL, *Helv. Chim. Acta* **43**, 1422 (1960).

Separation and partial purification of PTE and arabanase from *P. digitatum* culture filtrate has been achieved and it has not so far been possible to separate macerating activity from PTE activity. It was found that on increasing both the concentration and purity of the PTE the macerating activity showed, within the limits of the assay, a comparable increase; furthermore the purest PTE fractions ran as a single band on disc electrophoresis in polyacrylamide gels. The arabanase containing fractions showed no macerating activity, as assayed on a semi-quantitative basis by assessing the degree of softening of discs of orange peel placed in different dilutions of the enzyme for a standard time.

The separation and partial purification of the enzymes was carried out, after a preliminary fourfold concentration by freeze drying and reconstitution in a suitably reduced volume, by two fractional precipitations with ammonium sulphate followed by chromatography on ion-exchange cellulose.

TABLE 1. FIRST AMMONIUM SULPHATE FRACTIONATION OF *Penicillium digitatum* CULTURE FILTRATE

Fraction	Pectin transeliminase		Arabanase		Maceration
	Activity (0·01 absorptivity units/ml/min)	Specific activity (units activity per mg protein)	Activity (μg arabinose liberated/min)	Specific activity (units activity per mg protein)	Degree of softening
Culture filtrate	62	18·8	26·7	8·1	+
Concentrated culture filtrate	274	20·8	136·7	10·4	+++
$(NH_4)_2SO_4$ saturation					
0·2	0	—	5·2	2·3	0
0·4	300	97·4	346·7	112·6	+++
0·5	2060	647·8	640·0	201·6	+++*
0·6	302	95·0	110·7	34·8	+++
0·8	24	4·3	43·3	7·8	+
1·0	0	—	10·0	2·8	0
Residue (material not precipitated by saturated $(NH_4)_2SO_4$)	0	—	13·0		0

* Diluted 1 to 10, otherwise undiluted fractions were used.

Table 1 shows the distribution of PTE activity, arabanase activity and macerating activity in the first ammonium sulphate fractionation. The precipitate formed at 0·5 ammonium sulphate saturation was used as starting material in a second ammonium sulphate fractionation, the results of which are given in Table 2.

Solutions of the precipitates formed at 0·4 and 0·5 ammonium sulphate saturations in the second fractionation were, after desalting on Sephadex G25, passed through ECTEOLA cellulose to effect a separation of PTE and arabanase using a stepwise elution with sodium chloride as shown in Fig. 1.

The specific activity of PTE was increased from 18·8 units/mg protein in the culture filtrate to 4852 units/mg in the purest fraction (fraction 5) from the ECTEOLA cellulose column giving an overall 258-fold increase in specific activity for the purification process. Similarly the specific activity of the arabanase was increased from 8·1 units/mg protein in the culture filtrate to 495 units/mg protein in fraction 21 from the ECTEOLA column giving an overall 61-fold increase in specific activity.

Table 2. Second ammonium sulphate fractionation using the 0·5 saturation precipitate from Table 1

Fraction	Pectin transeliminase		Arabanase		Maceration
	Activity (0·01 absorptivity units/ml/min)	Specific activity (units activity per mg protein)	Activity (μg arabinose liberated/min)	Specific activity (units activity per mg protein)	Degree of softening
0·5 sat. $(NH_4)_2SO_4$ precipitated from first fractionation	2060·0	647·8	640·0	201·6	+++*
Saturation					
0·2	54	135·0	5·2		+
0·4	4120·0	710·3	1174·0	202·4	+++†
0·5	2618·0	808·0	55·0	17·1	+++*
0·6	82·0	49·4	0	—	+
0·8	0	—	0	—	0
1·0	0	—	0	—	0
Residue (material not precipitated by saturated $(NH_4)_2SO_4$)	0	—	0	—	0

* Diluted 1 to 10.
† Diluted 1 to 20. Otherwise undiluted fractions were used.

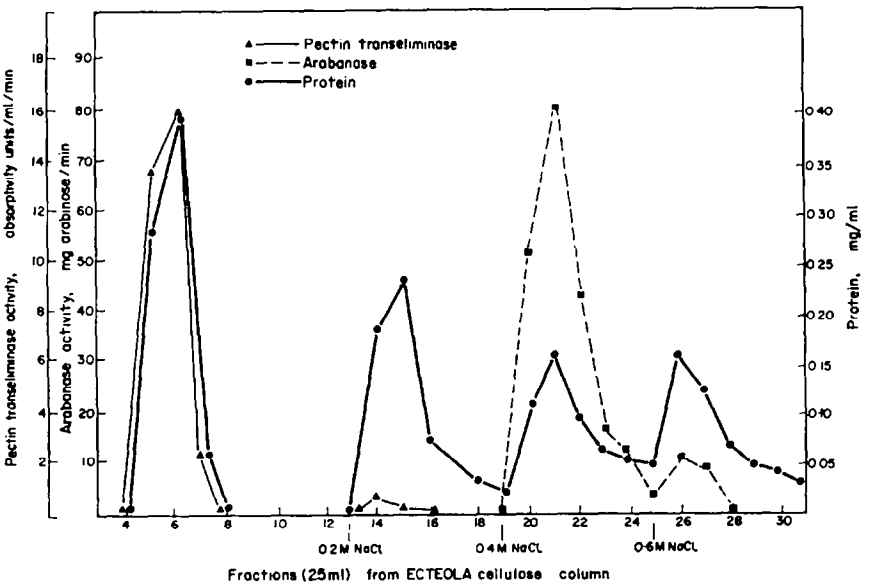

Fig. 1. The separation of arabanase and pectin transeliminase by column chromatography on ecteola cellulose.

25-ml fractions were eluted with water followed by increasing concentrations of NaCl. The NaCl marks on the base line of Fig. 1 indicate where each concentration is first eluted from the column.

Examination of the action of purified PTE on pectin by TLC gave no evidence of the formation of galacturonic acid or its dimer or trimer. This suggested that the enzyme is of the endo-type, giving rise to large oligomer units which do not move from the point of origin.

DISCUSSION

The results reported suggest that the macerating factor of *Penicillium digitatum* is identical with the PTE produced by this mould. It has not been possible by the methods applied to effect a separation of these activities. Further, the most highly purified PTE preparations ran as a single band on disc electrophoresis under the conditions used. While the estimation of macerating activity is difficult to carry out on an accurate quantitative basis there is some indication that macerating activity increases with increasing PTE concentration.

Chromatographic evidence suggested that the PTE of *P. digitatum* was an endo-enzyme since no galacturonic acid monomer could be detected in reaction mixtures by TLC. Preliminary experiments indicated that this enzyme was almost entirely specific for methylated pectin, since completely demethylated pectin was attacked at about 1/500th of the rate at which 50 per cent methylated pectin was attacked. There was also some indication that the rate of attack was proportional to the percentage methylation of the substrate.

The identity of macerating factor with endo-PTE is in agreement with the results of McClendon[14] using the commercial enzyme preparations Pectinol R.10 (Rhom and Haas Co.) and Pectinase (Nutritional Biochemicals Corp.), and with the views put forward by Bateman and Millar[10] on the nature of macerating factor. The work of Cole[16] casts doubt on the suggestion that the PTE component of the pectic enzyme complex of *P. digitatum* is responsible for maceration by the observation that maceration can occur in cultures grown under conditions which do not give PTE. In no case, however, was macerating activity reported to be absent where PTE was present. In the work reported here cultures grown on one medium only and under one set of aeration conditions were examined and all showed macerating and PTE activity. It is clear from the results in Tables 1 and 2 that on ammonium sulphate fractionation those fractions with the highest total PTE activity and specific activity show the greatest degree of softening of orange peel. Further, in the fractions from the ECTEOLA cellulose column, only those showing PTE activity produced maceration. In the first ammonium sulphate fractionation the PG activity appears in the 0·8 saturated fraction, well separated from PTE. It is not denied that more than one macerating system may operate in *P. digitatum* culture filtrates produced under varying medium and culture conditions. The results given above however, strongly indicate that, in culture conditions where PTE is produced, the presence of this enzyme is sufficient to account for the macerating activity of the culture filtrate.

It would be of interest to screen other organisms, which are known to macerate plant tissue, for endo-PTE and to apply the same separation procedure to the culture filtrates of these organisms as have been applied here. A preliminary investigation of *Aspergillus fonsecaeus* has shown that, as reported by Edstrom and Phaff,[17] this organism produces PTE. It has also been shown to invade oranges, producing noticeable softening.

Other pectic enzymes have been demonstrated in *P. digitatum* culture filtrate, for example exo-PG and PME, but it is thought that these do not play a key part in maceration, since enzymes of these types are also produced by *P. notatum* which does not macerate citrus tissue. Neither can these pectic enzymes be detected in the purified PTE fractions which show strong macerating activity.

EXPERIMENTAL

Growth Conditions of Penicillium digitatum *and* Penicillium notatum

The culture of *P. digitatum* was obtained from a mouldy orange and *P. notatum* strain was obtained from the Bath University of Technology culture collection. Both organisms were maintained on malt extract agar in

[16] A. L. J. Cole, Doctoral Thesis, Univ. Lond. (1967).

[17] R. D. Edstrom and H. J. Phaff, *J. Biol. Chem.* 239 (8) 2403 (1964).

universal bottles. In order to inoculate flasks of liquid media spore suspensions were prepared from 20-oz medical flat-bottle cultures on malt extract agar to give a spore concentration of approximately 10^6 spores/ml. One ml of this suspension was used to inoculate 75 ml of a malt extract liquid medium in a 250 ml conical flask. The flasks were shaken on a New Brunswick Gyrotary shaker at 25° using a 1 in. throw at 330 rev/min. After 3 days the cultures were bulked and filtered through Green's No. 904 paper on a Buchner funnel. The culture filtrate was freeze-dried in bulk in an Edwards Model 30 centrifugal freeze drier. The freeze-dried material from 4l. was then made up to 1 l. with distilled water prior to fractionation.

Fractionation Procedures

Ammonium sulphate fractionations were carried out by stirring into the enzyme solutions appropriate amounts of $(NH_4)_2SO_4$[18] by stepwise additions to give saturations of 0·2, 0·4, 0·5, 0·6, 0·8, and 1·0 at room temperature. The precipitates formed were centrifuged at 12,000 rev/min (23,000 *g*) for 20 min at 5° and redissolved in 100 ml of water. The solution containing the active precipitate (0·5 saturation) from the first fractionation was then reprecipitated with ammonium sulphate as before and each precipitate was taken up in 15 ml of water.

ECTEOLA cellulose fractionation was preceded by passage of an aqueous solution of the active fractions from the second $(NH_4)_2SO_4$ fractionation through Sephadex G25 to remove salts. The Sephadex was suspended in water and poured into a column 30 × 1·8 cm. 30 ml of material were put onto the column and eluted with water. 25-ml fractions were collected and assayed for PTE activity and salt content by a conductivity cell. The first two fractions, which were free of salt, contained 90 per cent of the PTE activity, and these were then put onto a column (45 × 2·5 cm) of ECTEOLA cellulose prepared in the following manner. 25 g of ECTEOLA cellulose was freed from fines by decantation and suspended in 500 ml of 0·5 N HCl for 1 hr. The cellulose was then filtered, washed with water till the filtrate was about pH 4·0–5·0, and suspended in 0·5 N NaOH for 1 hr and finally re-washed to *ca.* pH 7·0. The washed material was suspended in 500 ml of 0·5 M KH_2PO_4 washed by 0·01 M phosphate buffer (pH 7·5) till the pH of the filtrate was 7·5. Finally the ECTEOLA cellulose was washed in water.

The enzymes were eluted from the ECTEOLA cellulose first with 200 ml of water then 150 ml each of 0·1, 0·2, 0·4 and 0·6 M NaCl. 25-ml fractions were collected and each was assayed for arabanase, PTE, protein content and macerating activity.

Chromatographic Techniques

The soluble material resulting from the maceration of orange rind by *P. digitatum* culture filtrate was examined by TLC.[19] Plates were prepared with Kieselguhr G made up in 0·02 M sodium acetate and the samples were run in an ethyl acetate:isopropanol:water (65:23:12). Colour development was by aniline hydrogen phthalate (0·93 g of aniline and 1·66 g of phthalic acid in 100 ml of water-saturated *n*-butanol).

The degradation of citrus pectin by PTE was examined on a silica gel TLC.[20] The silica gel was made up in 0·07 M NaH_2PO_4 and the samples were run in a butanol:ethanol:0·1 M phosphoric acid (1:10:5) solvent system. The spots of pectin and galacturonic acid were developed by spraying with conc. H_2SO_4 in ethanol (1:1 v/v), which would also have shown up oligomers had these been present.

Plate Assay Techniques for PME and PG

The methods used were those developed by Dingle *et al.*[21] For the assay of PME, the agar contained Kodak citrus pectin 1·0%, salicylanilide 0·01%, agar 2·0% dissolved in distilled water to 100%. The pH was adjusted to 6·0 with NaOH. After autoclaving, sufficient methyl red in ethanol was added to the solution to give a final concentration of 0·01%. The pH was then readjusted with NaOH if necessary to discharge any trace of red colour of the indicator.

For the PG assay the agar contained sodium polypectate 1·0% (prepared from Kodak citrus pectin by the method of Jermyn and Tompkins[22]), salicylanilide 0·01%, ammonium oxalate 0·7%, agar 2·0% dissolved in 0·2 M acetate buffer, pH 5·0, to 100%.

Both media were autoclaved at 15 lb/in^2 pressure for 15 min and poured into Petri dishes in layers 3 mm thick. Holes were cut in the agar with an 8 mm cork-borer and 0·1 ml of enzyme solution was added to the well. The plates were incubated for 20 hr. PME activity was detected by the presence of a red zone around the well, whereas PG activity was detected by the presence of a clear area surrounded by a white halo around the well after development with 5 N HCl. Though it is claimed that the zone diameter varies linearly with the log of the enzyme concentration these tests were only used qualitatively in this work.

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[19] E. STAHL and V. KALTENBACH, *J. Chromatog.* **5**, 351 (1961).
[20] YU. S. OVODOV, *J. Chromatog.* **26**, 111 (1967).
[21] J. DINGLE, W. W. REID and G. L. SOLOMONS, *J. Sci. Food Agri.* **4**, 149 (1953).
[22] M. A. JERMYN and T. G. TOMPKINS, *Biochem. J.* **47**, 437 (19650).

PTE Assay

The method of Albersheim and Killias[23] was used with slight modification. 0·1 ml of enzyme was allowed to act on 3 ml of 0·5% pectin in 0·1 M citrate phosphate buffer at pH 5·2 and room temperature in a 1 cm silica cuvette. The increase in u.v. absorption at 235 nm was recorded. In the original method the substrate was cleared enzymically, but in this present work it was found more convenient to clear the substrate by centrifuging at 18,000 rev/min (40,000 g) for 1 hr at 18° One unit of trans eliminase activity was that amount of enzyme which caused an increase in optical density at 235 nm of 0·01 absorptivity units in 1 min under the conditions described above. It had previously been confirmed that the products of PTE action on pectin showed an absorption maximum at 235 nm when scanned on a Unicam SP 800 recording spectrophotometer.

Arabanase Assay

Arabanase was estimated by measuring the arabinose liberated from 3 ml of a 10 mg/ml beet araban substrate in 0·1 citrate phosphate buffer at pH 3·9 and 38° by 1 ml of enzyme. Beet araban for use as substrate was prepared by the method of Jones and Tanaka.[24] The liberated arabinose was measured by the method of Tracey.[25] One unit of arabanase activity was that amount of enzyme which liberated 1 μg of arabinose in 1 min under the conditions described.

Protein Estimations

Protein was estimated by the method of Lowry *et al.*[26]

Macerating Activity

Macerating activity was estimated by placing discs of orange rind cut with a number 5 cork-borer into solutions of enzyme for 1 hr at room temperature. Maceration was scored at the highest possible dilution on the following arbitrary scale.

±	very slight softening compared with control
+	softening
+ +	extreme softening
+ + +	partial disintegration

Electrophoresis

Fractions were examined for homogenity by disc electrophoresis on polyacrylamide gels at pH 9·5 according to the technique of Davis and Ornstein[27] using the apparatus of Shandon. Gels were run at 5 m.a. per tube and stained for protein with Naphthalene black 12B.

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