A Comparison of the Polygalacturonases Produced *in vivo* and *in vitro* by *Penicillium expansum* Thom.

By T. R. SWINBURNE

*Plant Pathology Research Division, Ministry of Agriculture for Northern Ireland
and School of Agriculture, Queen's University, Belfast*

AND M. E. CORDEN

*Botany and Plant Pathology Department, Oregon State University,
Oregon, U.S.A.*

(Accepted for publication 23 August 1968)

SUMMARY

Polygalacturonase (PG) was produced by *Penicillium expansum* in rotted apples and in liquid culture medium containing pectin; acetone precipitates of the enzyme were prepared from both sources. The PG extracted from apples (*in vivo* PG) behaved as an endo-PG and degraded sodium polypectate to tetragalacturonic acid. PG from culture filtrates (*in vitro* PG) before purification had quite different properties, the final hydrolysis product being monogalacturonic acid. PG from both sources was purified by ion-exchange chromatography. The properties of some purified PG preparations were quite different from those of the crude material. None of the purified *in vitro* PG released monogalacturonic acid from sodium polypectate, whilst some preparations from both sources had identical endo-PG properties after purification. Changes in the properties of endo-PG produced *in vivo* and *in vitro* were apparent during purification and a series of distinct endo-PG's were obtained which yielded unresolved oligomers, tri- or tetragalacturonic acid as final hydrolysis products. It is suggested that the changes in behaviour of the various PG preparations during purification were brought about by changes in the molecular configuration of the enzyme, and that such changes could account for some of the variability between enzymes produced *in vivo* and *in vitro*.

INTRODUCTION

Pectic glycosidases (poly α -1,4-galacturonide glycano hydrolase E.C. 3.2.1.15) have been classified according to their substrate specificity and the position of the bonds which they hydrolyse. Thus polygalacturonases (PG) preferentially attack pectic acid whilst polymethylgalacturonases (PMG) preferentially attack pectins of high methoxyl content. Endo-PG and endo-PMG were further defined as hydrolysing the α -1,4-glycosidic bonds in the substrate at random, whilst exo-PG and exo-PMG hydrolyse the terminal link (Demain & Phaff, 1957). Poly α -1,4-D-galacturonide lyases (E.C. 4.2.99.3) have been similarly classified (Bateman & Millar, 1966). It has been inferred that pectic enzymes produced by the pathogen are involved in the development of plant disease in a variety of host/pathogen combinations (Husain & Kelman, 1959), particularly for soft rots of fleshy organs. Difficulties in assessing the individual role of these enzymes are primarily due to problems of extraction from host tissue, resulting

from inactivation by oxidised host polyphenols (Akinrefon, 1967), and this has frequently led to the use of *in vitro* produced enzymes in experimental studies. Differences between enzymes produced *in vivo* and *in vitro* have been frequently demonstrated (Bateman & Millar, 1966). *Botrytis cinerea* produces different types of enzymes in culture media at various pH values (Damle, 1952) and yet different enzymes on onion leaves (Hancock, Millar & Lorbeer, 1964). *Penicillium expansum*, which is responsible for considerable wastage in stored apple fruit, produced endo-polygalacturonase *in vivo* and endo-polymethyl-galacturonase *in vitro* where pectin was the sole carbon source (Cole & Wood, 1961). Oxidation of polyphenolic materials does not occur to any great extent in *P. expansum* rotted apples, and extracts have relatively high degrees of pectic enzyme activity (Cole & Wood, 1961). By using various separation techniques, particularly ion-exchange chromatography, isolation and purification of pectic enzyme mixtures produced simultaneously by some fungi has been achieved (Rexova-Benkova & Slezarik, 1966; McClendon & Kreisher, 1963; Endo, 1963), and the presence of distinct types of endo-PG demonstrated (McClendon & Kreisher, 1963; Endo, 1963). The formation and dissociation of enzyme complexes during ion-exchange chromatography has also been recorded (Swinburne & Corden, 1967). The objective of the present work was to separate the various PGs produced *in vivo* and *in vitro* by *Penicillium expansum* and to compare their properties.

METHODS

The isolate of *Penicillium expansum* used in this work came from a naturally rotted Bramley's Seedling apple obtained from Co. Armagh, Northern Ireland, and was maintained on 2% malt agar.

PGs produced *in vitro* were obtained from shake cultures grown in a modified Richards medium containing (g./l.): 4, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 5.0, NH_4NO_3 ; 2.5, KH_2PO_4 ; 0.02, ZnSO_4 ; 0.02, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$; 10, pectin N.F. (Sunkist Inc.); in tap water. The pH value of the medium increased from 4.2 to 4.7 after 6 days' incubation at 25°. The organism was removed by centrifugation and discarded. Toluene (0.1%, v/v) was added to the supernatant fluid as preservative and the supernatant fluid dialysed against running tap water for 18 hr. At each stage samples of the enzyme mixture were plated for sterility. Several methods for the precipitation of the PG from the dialysate were evaluated. The method finally adopted was to add slowly an equal volume of acetone at -20° to the dialysate at 0°. The mixture was allowed to stand for 15 min., the precipitate removed by centrifugation and redissolved in a minimum volume of water and stored at -20°. Approximately 83% of the PG, as determined viscometrically, was recovered.

Pectic enzymes produced *in vivo* were obtained from rotted cortical tissue removed from Bramley's Seedling apples held 15 days at 25° following artificial inoculation with *Penicillium expansum* by a cork borer method (Granger & Horne, 1924). The rotted tissue was first frozen to aid maceration and 2 kg. samples homogenized with 2 l. 0.5 N-NaCl containing 5% (w/v) polyvinylpyrrolidone (General Aniline and Film Co., Polyclar AT; Akinrefon, 1967). The homogenate was pressed through muslin and filtered through Whatman no. 1 paper until a clear solution was obtained. After adding toluene (0.1%, v/v) the filtrate was dialysed against running tap water for 18 hr. To obtain a suitably flocculent precipitate of protein from the dialysate with

acetone it was found necessary first to add NaCl (0.2 g./100 ml.) followed by 2 vol. acetone. After the precipitate had been redissolved in water, viscometric assay indicated that 88% of the original PG had been recovered.

Enzyme+substrate reaction mixtures contained 0.5% (w/v) sodium polypectate (Sunkist), 10% (v/v) enzyme sample in 0.1 M-sodium acetate buffer (pH 5.0) with 0.001% (v/v) Merthiolate (Eli Lilly and Co.) as a preservative. Controls contained boiled enzyme or an equivalent volume of water. For viscosity decrease assays 8 ml. samples of the reaction mixture previously equilibrated to 37.5° were rapidly transferred to Cannon-Fenske viscometers (size 200) and the time taken (t) for the relative viscosity to fall to 50% ($\eta_{R_{50}}$) of the initial value determined by the method of Wood (1955). Units of PG activity were calculated as $1000/t$. Release of reducing groups in various enzyme+substrate reaction mixtures incubated at 37.5° were determined colorimetrically with dinitrosalicylic acid reagent (Miller, 1959) and calculated as μ g. galacturonic acid/ml. The percentage of galacturonic acid anhydride units in the sample of sodium polypectate used throughout these studies was estimated with carbazole reagent (Cole & Wood, 1961) to be 63.3% (w/w). The quantity of galacturonic acid that would be present in the enzyme+substrate reaction mixture when the hydrolysis was complete was calculated from this, and the hydrolysis at any stage was calculated as % of the galacturonic acid equivalent found by reducing-group methods of this quantity. For determinations of % hydrolysis at $\eta_{R_{50}}$ viscometric and reducing-group assays were made simultaneously on the same reaction mixture.

Hydrolytic products in the reaction mixtures were identified chromatographically (Young & Corden, 1964). Reference spots of authentic monogalacturonic acid were included on each paper. Other oligomers were identified by their R_f values, and the identification was checked by calculating the R_m value, where $R_m = \log ([x/R_f]-1)$ (Bate-Smith & Westall, 1950). A linear relationship between R_m and the number of galacturonic acid residues in each oligomer to pentagalacturonic acid was found.

Molecular exclusion chromatography of various PG preparations were done on columns of Sephadex G-200 (Pharmacia Ltd.) equilibrated with 0.1 M-sodium acetate buffer (pH 5.0). A column 42 $\times$ 2.5 cm. was prepared and fitted with a water jacket which enabled separations to be carried out at 10°. Samples containing PG were brought to approximately 5% (w/v) sucrose to increase their density and 3 ml. samples were layered by syringe pipette on top of the column beneath the eluant head. The column was eluted with the buffer at a rate of approximately 10 ml./hr and 4.4 ml. fractions collected.

Ion-exchange chromatography of the various PG preparations were done on columns of either diethylaminoethylcellulose (DEAE-cellulose; Whatman D.E. 52) or carboxymethylcellulose (CM-cellulose; Whatman CM. 52). After precycling the exchangers according to the manufacturers' instructions, DEAE cellulose was equilibrated with tris-HCl buffer (pH 7.0; $I = 0.05$) and CM cellulose was equilibrated with 0.01 M-sodium acetate buffer (pH 5.0). Columns 45 to 50 cm. $\times$ 2 cm. were prepared from each of the exchangers, and 10 ml. samples containing PG applied. Eluant was delivered, at room temperature, to the column by a peristaltic pump at a flow rate of approximately 80 ml./hr and consisted of buffer with a linear gradient of sodium chloride concentration. Fractions of 10.8 ml. were collected.

PG in the fractions collected from the various columns was assayed by a cup-plate method (Dingle, Reid & Solomons, 1953). A dilution series of the enzyme applied to

the column was included on each plate, enabling the relative % PG concentration of each fraction and the over-all recovery to be estimated. The presence of sodium chloride in enzyme preparations did not alter the size of the halos they produced in this assay.

Fractions within individual peaks of PG eluted from the various columns were bulked and concentrated to approximately the volume of the original sample by dialysis against Carbowax 20 M. in Visking tubing at 2°. The concentrates were then stored at -20°.

Protein concentrations were estimated by determining the extinction at 260 and 280 m μ (Beckman D.B.-G.) and reference to the data of Warburg & Christian (1942).

RESULTS

Properties of the crude in vivo and in vitro PG preparations

The crude *in vitro* PG contained 0.10 unit PG/ μ g. protein and the *in vivo* preparation 0.36 unit/ μ g. protein, as determined viscometrically with sodium polypectate as substrate. When pectin was used as the substrate the viscometric activities of both preparations were approximately 5% of these values, indicating that PMG was not present in either.

No enzymes of the transeliminase type were detected by the micromethod of Ayers, Papavizas & Diem (1966) in either preparation incubated with pectin or sodium polypectate at pH 5.0 or 8.0.

Table 1. *The hydrolysis of sodium polypectate by crude in vivo and in vitro PG of Penicillium expansum and the nature of the hydrolytic products*

Time (hr)	Crude <i>in vivo</i> PG							Crude <i>in vitro</i> PG						
	% Hydrolysis	Hydrolytic products*						% hydrolysis	Hydrolytic products*					
		1	2	3	4	5	FR		1	2	3	4	5	FR
1	40.6	—	—	+	+	++	+	64.9	+	±	±	+	+	+
4	50.8	±	—	+	++	+	+	75.1	+++	—	—	—	—	±
8	50.1	.	.	.	.	.	.	78.2	.	.	.	.	.	.
10	.	+	—	+	++	±	+	.	+++	—	—	—	—	—
24	52.0	+	—	+	+++	—	—	92.2	+++	—	—	—	—	—

* Columns 1 to 5 mono- to penta-galacturonic acid; FR, unresolved oligomers; —, none detected; ±, trace; ++, relative concentration.

The hydrolysis of sodium polypectate by both enzyme preparations was followed over a 24 hr period, and the nature of the breakdown products was determined (Table 1). Monogalacturonic acid was the principal hydrolytic product released by the *in vitro* PG and virtually the only compound detectable after 4 hr incubation. The hydrolysis by the *in vitro* PG had reached 92% after 24 hr and would probably have been completed in a longer time. With the *in vivo* PG, however, only slight increase in hydrolysis occurred after 4 hr incubation when approximately half the available substrate bonds had been hydrolysed. Monogalacturonic acid in small quantities was detected at the later stages of hydrolysis, but the principal breakdown product was tetragalacturonic acid, with a small amount of trigalacturonic acid found at all stages (Table 1).

The difference between the crude *in vitro* and *in vivo* PG was further illustrated by

the values of the % hydrolysis of sodium polypectate at ηR_{50} for both enzymes (Table 4). Thus crude *in vitro* PG hydrolysed more than three times as many bonds as the crude *in vivo* PG to achieve the same reduction in viscosity.

Single peaks of PG were separated from both crude *in vivo* and *in vitro* preparations on Sephadex G. 200 (Fig. 1). The elution volumes of PG from both sources were almost identical, the average of duplicate experiments being 154 ml. for *in vitro* PG and 156 ml. for *in vivo* PG. Approximately quantitative recovery of PG was obtained from both preparations. Non-enzyme protein was eluted immediately following the void volume of the column and after the PG peaks from both crude preparations (Fig. 1). Rechromatography of the PG within each peak on Sephadex G. 200 resulted in elution at the same volume for each.

Fig. 1. Elution of PG produced *in vitro* and *in vivo* by *Penicillium expansum* from a column of Sephadex G. 200. PG activity is expressed as a % of the sample applied; —▲—, *in vivo* PG; —△—, *in vitro* PG. Extinction values at 280 m μ of fractions shown as —●— for *in vivo* preparation, —○— for *in vitro* preparation.

DEAE-cellulose chromatography of crude *in vivo* and *in vitro* PG

None of the PG in the *in vivo* preparation was retained by DEAE-cellulose, but was eluted in a single peak with the void volume (Fig. 2). Most of the protein was adsorbed and eluted as a single peak after the PG. In three separate experiments 74–75% of the *in vivo* PG applied to the columns was recovered, representing about a 200-fold purification. Likewise the PG in the crude *in vitro* preparations passed through DEAE-cellulose columns with the void volume and was eluted as a single peak whilst most of the protein was adsorbed and eluted at a higher salt concentration (Fig. 2). Recovery of *in vitro* PG from these columns was somewhat variable, the average of three experiments being 101%, representing a threefold purification.

After concentrating the preparations eluted from DEAE-cellulose of *in vitro* and *in vivo* PG, henceforward referred to as *in vitro* and *in vivo* DEAE-PG, respectively, to their original volume, they were separately applied to the Sephadex G. 200 column. The *in vitro* DEAE-PG was eluted in a single peak with an elution volume of 154 ml., which was identical to that of the crude *in vitro* PG. The *in vivo* DEAE-PG was similarly eluted as a single peak with an elution volume of approximately 157 ml. which corresponded closely to that obtained for the crude enzyme. The % hydrolysis with time of sodium polypectate by *in vitro* and *in vivo* DEAE-PG was followed, the nature of the hydrolytic products determined, and the value of the percentage hydrolysis at ηR_{50} was obtained (Table 2).

Fig. 2. Purification of PG produced *in vitro* and *in vivo* by *Penicillium expansum* on columns of DEAE-cellulose eluted with Tris-HCl buffer pH 7.0 and a linear gradient of sodium chloride. A, *in vivo* preparation; B, *in vitro* preparation. —▲—, PG activity expressed as a % of the sample applied to the column; —○—, extinction value at 280 mμ. Approximate salt concentrations shown by solid line.

Table 2. The hydrolysis of sodium polypectate by *in vivo* and *in vitro* DEAE-PG of *Penicillium expansum* and the nature of the hydrolysis products

Time (hr)	<i>In vivo</i> DEAE-PG							<i>In vitro</i> DEAE-PG						
	% Hydrolysis	Hydrolytic products*						% Hydrolysis	Hydrolytic products*					
		1	2	3	4	5	FR		1	2	3	4	5	FR
1	45.9	—	±	±	±	±	+++	40.1	—	—	—	—	—	+++
4	55.4	—	±	±	±	+	++	52.1	—	—	±	±	+	++
10	60.0	—	±	±	++	++	+		.	.	.	.	+	.
24	63.0	—	±	+	+++	±	±	60.6	—	—	±	++	++	+

* Columns 1 to 5 mono- to penta-galacturonic acid; FR, unresolved oligomers; —, no detected; ±, trace; ++, relative concentration.

The final % hydrolysis of the substrate achieved by the *in vivo* DEAE-PG after 24 hr was higher than with the crude *in vivo* PG, although the principal hydrolytic products that accumulated, namely tetra- with a little tri-galacturonic acid, were the same for both preparations. Monogalacturonic acid was not detected at any stage of the hydrolysis, although a trace of digalacturonic acid was present throughout. There was no significant difference between the values of the % hydrolysis at the point where 50% viscosity loss occurred for crude *in vivo* PG and for *in vivo* DEAE-PG (Table 4).

The properties of the *in vitro* DEAE-PG were however quite different from those of the original crude material. The degree of hydrolysis by *in vitro* DEAE-PG after 24 hr was very much less than that obtained with the crude *in vitro* PG (Table 2) and the principal hydrolytic products were tetra- and penta-galacturonic acids with a small quantity of trigalacturonic acid; monogalacturonic acid was not detected at any stage. This difference was also reflected in the % hydrolysis at ηR_{50} , the value for the *in vitro* DEAE-PG was less than half that of the crude *in vitro* PG (Table 4). This value was also significantly different from that obtained with crude *in vivo* PG and the *in vivo* DEAE-PG in spite of the similarity between the hydrolytic products released by these three enzyme preparations (Table 4).

Fig. 3. Purification of PG produced *in vivo* and *in vitro* by *Penicillium expansum* on columns of CM-cellulose eluted with 0.01 M-sodium acetate (pH 5.0) and a linear gradient of sodium chloride. A, *in vivo* preparation; B, *in vitro* preparation. —▲—, PG activity expressed as a % of the sample applied to the column; —○—, extinction values at 280 m μ . Approximate salt concentration shown as a solid line.

CM-cellulose chromatography of crude *in vivo* and *in vitro* PG

All the PG in the crude *in vivo* enzyme preparation was adsorbed on CM-cellulose columns, and eluted as a single peak by approximately 0.3 M-NaCl, whilst most of the other protein was eluted at or near the void volume of the column (Fig. 3). In three separate experiments an average of 94% of the crude *in vivo* PG applied to these columns was recovered, representing an approximately 100-fold purification after concentration to approximately the original volume (referred to as *in vivo* CM-PG).

All the PG in the crude *in vitro* enzyme preparation was also adsorbed on CM-cellulose, but was eluted in two separate peaks (Fig. 3). The first peak was eluted by approximately 0.3 M-NaCl and contained an average of 75% of the PG recovered, whilst the second peak containing an average of 25% of the recovered PG was eluted by 0.35 M-NaCl. The recovery of crude *in vitro* PG applied to such columns was consistently high, the average of four separate experiments being 118%. Most of the inactive protein in the crude *in vitro* PG preparation was eluted at low salt concentrations (Fig. 3). The fractions within each peak were concentrated to approximately the volume of the original sample. The PG in the first peak, referred to as *in vitro* CMI-PG, represented an eightfold purification, whilst the PG in the second peak, referred to as *in vitro* CMII-PG, represented a fourfold purification of the original crude material.

The *in vivo* CM-PG was eluted from the Sephadex G. 200 column as a single peak in a volume of approximately 145 ml., which was less than the elution volume of the original crude enzyme. Similarly, the *in vitro* CMI-PG was eluted as a single peak in 141 ml., which was less than the crude enzyme, whilst *in vitro* CMII-PG was eluted as a somewhat broad peak in a volume of between 158 and 167 ml.

Table 3. *The hydrolysis of sodium polypectate in vivo CM-PG and in vitro CMI-PG*

<i>In vivo</i> CM-PG							<i>In vitro</i> CMI-PG							
Time (hr)	% Hydro- lysis	Hydrolytic products*					% Hydro- lysis	Hydrolytic products*						
		1	2	3	4	5		FR	1	2	3	4	5	FR
1	55.3	.	.	.	.	.	.	.	—	—	—	—	—	+++
4	56.3	—	±	±	+	++	+	37.8	.	.	.	.	.	.
10	57.6	—	+	++	+	±	—		.	.	.	.	.	.
24	71.0	—	+	+++	±	—	—	68.0	+	±	±	++	—	+

* Columns 1 to 5 mono- to penta-galacturonic acid; FR, unresolved oligomers; —, none detected; ±, trace; ++, relative concentration.

The *in vivo* CM-PG achieved a considerably higher % hydrolysis of sodium polypectate in 24 hr (Table 3) than did the crude *in vivo* PG (Table 1). Penta- and tetra-galacturonic acid were the main hydrolytic products detected after 4 hr incubation, but at all subsequent stages of the hydrolysis only trigalacturonic remained in quantity, with some digalacturonic acid, whereas only traces of tetragalacturonic acid could be detected (Table 3). The percentage hydrolysis of sodium polypectate at ηR_{50} was twice that obtained with the crude *in vivo* PG and this value was also significantly different from both crude *in vitro* PG and *in vitro* DEAE-PG (Table 4).

Although the *in vitro* CMI-PG hydrolysed sodium polypectate more completely than the *in vitro* DEAE-PG (Table 2) the final % of hydrolysis was still considerably less than had been achieved by the crude *in vitro* PG. Tetragalacturonic acid was the principal hydrolytic product after 24 hr incubation. In contrast with *in vitro* DEAE-PG, monogalacturonic acid was present after 24 hr. The value for the % hydrolysis of sodium polypectate at ηR_{50} by *in vitro* CMI-PG was not significantly different from those obtained with *in vitro* DEAE-PG or *in vivo* DEAE-PG (Table 4).

The *in vitro* CMII-PG preparation had relatively low activity and consequently it was necessary to follow the hydrolysis of sodium polypectate over 48 hr. No hydrolytic products were detected until after 24 hr of incubation, when 37.9% hydrolysis

had occurred, but only unresolved oligomers were detected. During the next 24 hr only a slight increase in hydrolysis to 43 % was obtained, and no further products were detected chromatographically. The value for the % hydrolysis at ηR_{50} was the lowest obtained and significantly different from all other preparations (Table 4).

Table 4. *The percentage hydrolysis of sodium polypectate by various enzyme preparations at the point where half the initial relative substrate viscosity was lost*

Source	Preparation	% hydrolysis	Fiducial limits (5 %)
<i>In vivo</i>	Crude precipitate	1.7	± 0.38
	DEAE preparation	1.9	± 0.71
	CM preparation	4.2	± 0.32
	DEAE/CM preparation	2.0	± 0.08
<i>In vitro</i>	Crude precipitate	6.1	± 1.18
	DEAE preparation	2.8	± 0.16
	CMI preparation	1.8	± 0.38
	CMI preparation	0.34	± 0.81
	DEAE/CM preparation	1.8	± 0.10

Rechromatography of in vivo and in vitro DEAE-PG on CM cellulose

After concentration to approximately their original volume, 10 ml. of *in vivo* and *in vitro* DEAE-PG were separately applied to CM-cellulose columns which were eluted with a linear gradient of NaCl. All the PG in the *in vivo* DEAE preparation was adsorbed on these columns and was eluted as a single peak by approximately 0.3 M-NaCl, corresponding exactly to the crude *in vivo* PG. Approximately 105 % of the *in vivo* DEAE-PG applied to CM cellulose columns was recovered (referred to as *in vivo* DEAE/CM-PG).

The PG in the *in vitro* DEAE preparation was also adsorbed on CM cellulose, and was similarly eluted as a single peak by approximately 0.3 M-NaCl. No PG peak corresponding to *in vitro* CMIIPG was obtained. In two experiments 72 and 82 % of the *in vitro* DEAE-PG applied to CM cellulose columns was recovered (referred to as *in vitro* DEAE/CM-PG). After reconcentrating both the *in vivo* and *in vitro* DEAE/CM-PG preparations to their original volumes they were separately applied to the Sephadex G. 200 column. The *in vivo* DEAE/CM-PG was eluted in a single peak in a volume of approximately 150 ml., which was only slightly less than that obtained for the crude *in vivo* PG. *In vitro* DEAE/CM-PG was also eluted as a single peak with an elution volume identical to the original crude material, namely 154 ml. There were no significant differences in values for % hydrolysis at ηR_{50} between either of the DEAE/CM preparations nor were they different from *in vitro* CMI-PG, *in vivo* DEAE-PG or crude *in vivo* PG (Table 4).

A comparison of cup-plate and reducing-group assays

Because the crude *in vitro* PG rapidly liberated monogalacturonic acid from sodium polypectate the presence of an exo-PG was suspected, but no exo-PG activity was observed in any of the purified preparations. The possibility that exo-PG was present in enzyme fractions but had escaped detection by cup-plate assay was therefore investigated. Fractions eluted from a CM-cellulose column following the application of crude *in vitro* PG were assayed both by the cup-plate method and by determining the

reducing groups released from 1 ml. of 0.5% (w/v) sodium polypectate in 1 hr at 35.5° by 0.1 ml. of each enzyme fraction. Cup-plate assay showed PG activity in two peaks as usual; 70% was recovered in the first peak (*in vitro* CMIPG) and approximately 33% was recovered in the second (*in vitro* CMII-PG). Only those fractions shown to contain PG by cup-plate assay liberated reducing groups from sodium polypectate. Reducing-group assay also showed that 70% of the applied PG was recovered in the first peak. However, the fractions in the second peak only contained approximately 3% of the applied PG, giving an over-all recovery of 73%. Thus the activity of *in vitro* CMII-PG relative to the crude *in vitro* PG as measured by reducing-group assay was only one tenth the cup-plate assay value. In a further comparison the activity of *in vitro* CMIPG relative to the crude *in vitro* PG was found to be 3.3 and 5.3% by cup-plate and viscometric assays, respectively but only 0.3% by measurement of the rate of release of reducing groups.

DISCUSSION

The differences between the crude pectic enzymes produced *in vivo* and *in vitro* by the isolate of *Penicillium expansum* used in these studies were in the chemical bond hydrolysed rather than in the substrate attacked. Cole & Wood (1961), however, found that other isolates of *P. expansum*, produced PMG *in vitro* and PG *in vivo*. Differences in bond specificity for these enzymes is similar to those found between PG produced *in vivo* and *in vitro* by *Rhizoctonia solani* (Bateman, 1963); thus crude *in vivo* *P. expansum* PG did not degrade sodium polypectate further than tetragalacturonic acid, whilst crude *in vitro* PG completely hydrolysed the substrate to monogalacturonic acid. It is doubtful whether all or even part of the PG activity observed in the crude *in vitro* preparation can be attributed to exo-PG. Liberation of monogalacturonic acid at an early stage in hydrolysis has been taken to be evidence for the presence of exo-PG (e.g. Hancock *et al.* 1964). However % hydrolysis at ηR_{50} for crude *in vitro* PG of *P. expansum* was considerably lower than for exo-PG produced by *Coniothyrium dipodiella* with pectic acid as the substrate (Endo, 1964). Whilst the value obtained would obviously depend on the initial chain length of the substrate, the figure of 6.1% is probably too low for exo-PG alone. This value could represent the net result of the combined action of exo- and endo-PG; but the inability to distinguish more than one enzyme by molecular exclusion-chromatography of the crude material and the absence of a distinct exo-PG by ion exchange chromatography suggests that only one PG was present. From thermal inactivation studies Bateman (1963) also concluded that, *in vitro*, *R. solani* produced only one PG, whose properties closely resembled the crude *in vitro* PG of this study. No difficulties of classification pertain to the crude *in vivo* PG where all the observed activity can be attributed to endo-PG which did not significantly degrade the substrate beyond tetragalacturonic acid. A similar endo-PG has been described for yeasts (Luh & Phaff, 1954).

One of the most interesting features of this study was that purification of both crude *in vitro* and crude *in vivo* PG of *Penicillium expansum* sometimes yielded enzymes whose properties did not correspond with the original material. Thus none of the ion-exchange cellulose preparations of *in vitro* PG liberated significant quantities of a monogalacturonic acid. The high recovery values obtained indicate that no PG was lost during purification, for example by inactivation of exo-PG in the original crude material. Consistently high recoveries of PG from *Fusarium oxysporum* f. sp. *lyco-*

persici applied as a crude culture filtrate to DEAE-cellulose columns were also obtained by T. R. Swinburne & M. E. Corden (private communication). From a comparison of the results of the various PG assay methods it was apparent that the cup-plate technique was directly comparable with viscometric assay, but not necessarily with reducing-group assay. The cup-plate assay is relatively more sensitive to endo-PG than to exo-PG, and, moreover, enzymes with low percentage hydrolysis at ηR_{50} , such as *in vitro* CMII-PG, produce proportionately larger halos than endo-PGs, such as *in vitro* CMI-PG, which hydrolyse the substrate more completely. This suggests that the differences between crude *in vitro* PG and *in vitro* DEAE-PG and CMI-PG might be due to changes in the properties of the enzyme molecule. If such changes resulted in an over-all increase in the viscometric activity the recovery values on a cup-plate assay would be high. The stability of PG at the pH value of the DEAE-cellulose columns is relatively low (Bateman, 1963) and some inactivation of *in vivo* PG may have taken place. If *in vitro* PG were also inactivated at this pH value then the over-all recovery on DEAE-cellulose might have been even higher if this effect could have been avoided. With one exception, all changes in properties of enzyme preparations resulted in a decrease in % hydrolysis at ηR_{50} . The exception to this was *in vivo* CM-PG, where a marked increase was obtained, and the final hydrolytic product was also changed from tetra- to tri-galacturonic acid. The somewhat low recovery values obtained for *in vivo* PG on CM-cellulose may be related to enzyme inactivation, but the lower sensitivity of the cup-plate assay with such enzymes may also have affected the results.

Most preparations of *Penicillium expansum in vivo* and *in vitro* PG had similar properties. Thus crude *in vivo*-PG, DEAE-PG, DEAE/CM-PG, *in vitro* CMI-PG and DEAE/CM-PG were virtually identical in properties and might reasonably be assumed to be identical enzymes. The remaining preparations, all with properties of endo-PG, were however quite distinct from these and from each other. The simultaneous production of distinct forms of endo-PG has been described for various fungi (McClendon & Kreisher, 1963; Endo, 1963). The three forms of endo-PG separated from bran cultures of *Coniothyrium diplodiella* also differed in the relative ability to reduce substrate viscosity in relation to the number of bonds hydrolysed (Endo, 1963). The existence of forms of endo-PG with different properties shows that some PGs are, paradoxically, 'more random' in their hydrolytic action than others, a similar situation to that described for cellulases (Nisizawa, Hashimoto & Shibata, 1963). If the hydrolysis of $\alpha 1:4$ -glycosidic bonds in pectate substrates by endo-PG were truly random the percentage hydrolysis at ηR_{50} for most endo-PG preparations would be similar, since the number of bonds hydrolysed at ηR_{50} is a small proportion of the total. That this is not so is apparent from this and other studies (Endo, 1963).

The cause of the changes in enzyme properties during purification cannot be ascertained from the experiments reported here. Previous experiments with PG produced by *Fusarium oxysporum* f. *lycopersici* suggested that dissociation phenomena took place on DEAE-cellulose, as indicated by Sephadex G200 separation of the products (Swinburne & Corden, 1967). Whilst some slight differences were observed in elution volume on Sephadex G200 of *in vitro* and *in vivo* PG of *Penicillium expansum* following ion-exchange cellulose chromatography, these were much less than those observed with the *Fusarium* PG. The changes in property could have resulted either from alterations in the secondary or tertiary structure of the enzyme molecule as a result of the ionic conditions of separation (Steinburg & Mihalyi, 1957), or, less

probably, from the presence of substances which modified the mode of action of the enzyme. The explanation of these phenomena must await further experiments. If a range of properties can be evoked from one basic enzyme by appropriate treatments, the differences observed between *in vivo* and *in vitro* enzymes may also be attributable to different forms of the same enzyme. Bateman's (1963) work has shown that care must be taken in relating the properties of PG produced *in vitro* to the role of the enzyme in disease development *in vivo*, and this investigation suggests that care must be taken in extraction of enzymes produced *in vivo* to prevent changes from the native enzyme.

We wish to thank Mr M. Comer for his invaluable technical assistance throughout this work and Mr O'Neill (Biometrics Division, Ministry of Agriculture of Northern Ireland) for his advice on statistical problems.

REFERENCES

- AKINREFON, O. A. (1967). Improved extraction of polygalacturonase in apple tissue rotted by *Sclerotinia fructigena* Aderh. & Ruhl. *Rep. agric. hort. Res. Stn. Univ. Bristol* 1966, p. 168.
- AYERS, W. A., PAPAIVAS, G. C. & DIEM, A. F. (1966). Polygalacturonate transeliminase and polygalacturonase production by *Rhizoctonia solani*. *Phytopathology* 56, 1006.
- BATEMAN, D. F. (1963). Pectolytic activities of culture filtrates of *Rhizoctonia solani* and extracts of *Rhizoctonia*-infected tissues of bean. *Phytopathology* 53, 197.
- BATEMAN, D. F. & MILLAR, R. L. (1966). Pectic enzymes in tissue degradation. *A. Rev. Phytopath.* 4, 119.
- BATE-SMITH, E. C. & WESTALL, R. G. (1950). Chromatographic behaviour and chemical structure. I. Some naturally occurring phenolic substances. *Biochim. biophys. Acta* 4, 427.
- COLE, M. & WOOD, R. K. S. (1961). Pectic enzymes and phenolic substances in apples rotted by fungi. *Ann. Bot.* 25, 435.
- DAMLE, V. P. (1952). Enzymatic study of certain parasitic fungi. *J. India bot. Soc.* 31, 12.
- DEMAIN, A. L. & PHAFF, H. J. (1957). Recent advances in the enzymatic hydrolysis of pectic substances. *Wallerstein Lab. Commun.* 20, 119.
- DINGLE, J., REID, W. W. & SOLOMONS, G. L. (1953). The enzymatic degradation of pectin and other polysaccharides. II. The application of the cup-plate assay to the estimation of enzymes. *J. Sci. Fd Agric.* 4, 149.
- ENDO, A. (1963). Studies on pectolytic enzymes of molds. Part VI. The fractionation of pectolytic enzymes of *Coniothyrium diplodiella*, (2). *Agric. biol. Chem.* 27, 751.
- ENDO, A. (1964). Studies on pectolytic enzymes of molds. Part XI. Purification and properties of exo-polygalacturonase. *Agric. biol. Chem.* 28, 639.
- GRANGER, K. & HORNE, A. S. (1924). A method of inoculating the apple. *Ann. Bot.* 38, 212.
- HANCOCK, J. G., MILLAR, R. L. & LORBEER, J. W. (1964). Pectolytic and cellulolytic enzymes produced by *Botrytis allii*, *B. cinerea*, and *B. squamosa* *in vitro* and *in vivo*. *Phytopathology* 54, 928.
- HUSAIN, A. & KELMAN, A. (1959). Tissue is disintegrated. In *Plant Pathology*, ed. by J. C. Horsfall and A. E. Dimond, vol. 1, p. 143. New York: Academic Press.
- LUH, B. S. & PHAFF, H. J. (1954). Properties of yeast polygalacturonase. *Archs Biochem. Biophys.* 48, 23.
- MILLER, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analyt. Chem.* 31, 426.
- MCCLENDON, J. H. & KREISHER, J. H. (1963). Chromatography on cellulose phosphate of polysaccharide hydrolases from fungi. *Analyt. Biochem.* 5, 295.
- NISIZAWA, K., HASHIMOTO, Y. & SHIBATA, Y. (1963). Specificities of some cellulases of the 'random' type. In *Advances in Enzymic Hydrolysis of Cellulose and Related Materials*, ed. by E. T. Reese. New York: Pergamon Press.
- REXOVA-BENKOVA, L. & SLEZARIK, A. (1966). Isolation of extracellular pectolytic enzymes produced by *Aspergillus niger*. *Colln Czech. chem. Commun. Engl. Edn* 31, 122.

- STEINBURG, D. & MIHALYI, E. (1957). The chemistry of proteins. *A. Rev. Biochem.* **26**, 373.
- SWINBURNE, T. R. & CORDEN, M. E. (1967). Dissociation and recombination of a polygalacturonase complex during ion-exchange chromatography. *Nature, Lond.* **213**, 286.
- WARBURG, O. & CHRISTIAN, W. (1942). Isolierung und Kristallisation des Gärungsferments Enolase. *Biochem. Z.* **310**, 384.
- WOOD, R. K. S. (1955). Studies in the physiology of parasitism. XVIII. Pectic enzymes secreted by *Bacterium aroideae*. *Ann. Bot.* **73**, 2.
- YOUNG, R. J. & CORDEN, M. E. (1964). Paper chromatography of galacturonic acids to determine polygalacturonase activity. *Biochim. biophys. Acta* **17**, 124.