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PURIFICATION AND CHARACTERISATION OF POLYGALACTURONASES FROM A COMMERCIAL *ASPERGILLUS NIGER* PREPARATION

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Summary

The polygalacturonase (poly(1,4-α-D-galacturonide) glycanohydrolase, EC 3.2.1.15) activity of Pectinol is resolved into two fractions (E_1 and E_2) of about equal total activity on DEAE-cellulose. These fractions are purified from other pectinolytic enzyme activity by Sephadex G-75 chromatography. Both E_1 and E_2 reduce the viscosity of polygalacturonate by 50% after 7% of the glycosidic bonds are hydrolysed. Their activities are not affected by iodoacetate (1 mM) or EDTA (10 mM). E_1 and E_2 have different molecular weights (35 000 and 85 000, respectively) and different electrophoretic mobilities on sodium dodecyl sulphate polyacrylamide gels. Their pH (4.1 and 3.8 respectively) and ionic strength optima and specific activities also differ. Both enzymes are inhibited at similar rates by diethyl pyrocarbonate at pH 6 but only E_2 is protected from this inhibition by 2% (w/v) polygalacturonate. The rate of change of protein absorbance at 250 nm accompanying this inhibition, and the reversibility of this inhibition by neutral hydroxylamine indicate that histidine residues are essential for the activities of both E_1 and E_2. About 2 molecules of carbethoxyhistidine per subunit of E_2 and 0.6 molecules per subunit of E_1, are present in the completely inhibited enzymes.

Introduction

Commercial preparations of fungal pectinolytic enzymes are used as processing aids in the production of fruit and vegetable juices [1]. Knowledge about the enzyme activities present in these preparations is superficial [2]. The relative activities of polygalacturonate (pectate) and polymethylgalacturonate (pectin) depolymerising enzymes and pectin esterase (pectin pectyl-hydrolase, EC 3.1.1.11) are important determinants in the clarifying and viscosity reducing effects of these preparations [3—5]. The most common fungal pectate de-

polymerase is endopolygalacturonase [6], and endodepolymerases appear to be responsible for the phytopathogenicity of many fungi [7,8].

Endopolygalacturonases (poly(1,4-α-D-galacturonide) glycanohydrolases, EC 3.2.1.15) have been isolated from several fungi [9—22]; and the occurrence of different forms of this enzyme from the same source has often been reported [9—18]. Slight differences in enzyme action have been attributed to some of these different forms [9—14] but few data about their molecular properties exist. The existence of different conformers [13], polygalacturonase-substrate complexes [14], polygalacturonase-pectin esterase complexes [15] and isoenzymes could explain this phenomenon.

A study of the inhibitors of *Botrytis cinera* endopolygalacturonase indicated that an amino group is essential for activity [23]. This enzyme is also inhibited by compounds that react with sulphydryl and tyrosyl groups [15]. Patil and Dimond [24] observed that *Verticillium albo-atrum* endopolygalacturonase is inhibited by oxidised phenols and concluded that an amino (or possibly imino) group is essential for catalytic activity. This is in agreement with an earlier hypothesis [25] based on the inhibition of the yeast enzyme by nitrous acid. Photooxidation studies of the *Aspergillus niger* endopolygalacturonase provided indirect evidence that histidine is involved in the catalytic mechanism [26].

This paper reports the purification and characterisation of two endopolygalacturonases from *Aspergillus niger*. Using the reagent diethylpyrocarbonate [27], histidine is implicated as essential for the activity of both enzymes. A preliminary report of the early stages of this work has been presented [28].

Materials and Methods

Enzyme assays

Polygalacturonic acid (grade II, Sigma Chemical Co. Ltd., Kingston-upon-Thames, U.K.) and citrus pectin (Eastman-Kodak Ltd., Kirkby, Liverpool, U.K.) were slurried (50 g of each powder in 300 ml of solvent) in ethanol:water 80% (w/v) stirred for 30 min at 20°C, centrifuged (10 000 × *g* for 30 min) and the procedure repeated again in 80% ethanol and finally in acetone. This material was dried to constant weight in a vacuum oven at 40°C. Polygalacturonase activity was determined as the rate of release of reducing groups measured with ferricyanide [29] and using monogalacturonic acid (Sigma Chemical Co. Ltd.) as the standard. The assay solution consisted of 0.2 ml enzyme solution and 2 ml of 0.5% (w/v) polygalacturonic acid in 0.3 M acetate pH 4.0. One unit of polygalacturonase liberates 1 μmol of galacturonate per min at 30°C. The rate of release of reducing groups was proportional to added enzyme in the range 0 to 0.8 units. The rate of substrate viscosity reduction was measured by the flow times of the solution in an Ostwald-Fenske viscometer No. 200. The approcimate protein concentration of the enzyme solutions was measured by the absorbance at 280 nm, corrected for that at 260 nm [30]. Polymethylgalacturonase activity was determined using 0.5% pectin instead of 0.5% polygalacturonic acid in the assay medium.

Pectin lyase (poly(methoxygalacturonide) lyase, EC 4.2.2.10) activity was measured as the increase in absorbance at 235 nm of a solution consisting of

0.2 ml enzyme solution and 2 ml of 0.5% (w/v) pectin in 0.3 M McIlvaine buffer pH 6 [31] at 30°C. The molar extinction coefficient of the product is 5500 $1 \cdot mol^{-1} \cdot cm^{-1}$ [32], and 1 unit of activity produces 1 μmol of unsaturated product per min. Pectin esterase was assayed by titrating the carboxyl groups released from 5 ml of 0.5% pectin in 0.2 M NaCl at pH 4.5, using a TTT2 module, ABU11 autoburette and SBR3 recorder (Radiometer, Copenhagen, Denmark). One unit of activity releases 1 μmol of carboxyl groups per min at 22°C.

Purification of polygalacturonases

3.5 g of Pectinol (Rohm GmbH, D-61, Darmstadt, W. Germany) were dissolved in 15 ml of distilled water and dialysed against 3 × 4 l of distilled water for 16 h at 4°C. This solution was adjusted to pH 5.2 using a few drops of 0.5 M Na_2HPO_4 and applied to a column of DEAE-cellulose (microgranular DE-52, Whatman Biochemicals Ltd., Maidstone, Kent, U.K.) as described in the legend to Fig. 1. Two polygalacturonase fractions were recovered: E_1 which eluted in buffer 1 and E_2 which eluted in a similar position to pectin lyase. An almost identical elution profile was obtained from a second batch of Pectinol. Fractions 7—18 and 82—99 were pooled, dialysed against 4 × 20 vols. of distilled water for 18 h at 4°C and freeze-dried.

These powders were dissolved in 8 ml of 0.1 M acetate buffer pH 4.0 and 6 ml of each were applied separately to a column of Sephadex G-75 (Pharmacia Fine Chemicals, Uppsala, Sweden) as described in Fig. 2. E_2 was clearly resolved from pectin lyase and the latter was eluted at a similar position to E_1. The following molecular weight markers were used in gel filtration and gel electrophoresis experiments: 1. horse heart cytochrome *c*; 2. chicken egg albumin (5 × crystallised); 3. bovine albumin crystallised, purissa (1—3 from Koch-Light Labs. Lts., Colnbrook, Bucks, U.K.); 4. Soybean lipoxygenase type I; 5. porcine pepsin; 6. bovine heart lactate dehydrogenase (4—6 from Sigma Chemical Co. Ltd.); 7. ovotransferrin (a gift from Dr R.W. Evans. University of Bristol). The Sephadex G-75 column was calibrated according to the method of Andrews [33]. A sample (1 ml) of E_2 was subsequently chromatographed on an analytical column of Sephadex G-150 (47 × 1.2 cm) calibrated with the markers shown in Fig. 2 and soybean lipoxygenase (102 000). The active fractions from the G-75 columns were pooled, dialysed against 3 × 80 vols. of distilled water and freeze-dried.

Samples of these purified fractions were analysed electrophoretically on sodium dodecyl sulphate polyacrylamide gels as described earlier [34]. Logarithms of molecular weights were determined from the distances migrated by the method of Weber and Osborn [35].

Polygalacturonase inhibition by diethylpyrocarbonate

Diethylpyrocarbonate was obtained from BDH, Poole, Dorset, U.K.; N^{α}-acetylhistidine and hydroxylamine monohydrochloride grade I were from Sigma Chemical Co. Ltd. The diethylpyrocarbonate used in these experiments was 76% pure, calculated from the increase in absorbance at 240 nm after reaction of approximately 0.1 mM diethylpyrocarbonate with 10 mM *N*-acetylhistidine in 0.1 M phosphate buffer, pH 6.0 [27,36].

Diethylpyrocarbonate solutions were prepared just prior to use by dilution

with cold absolute ethanol. The difference in absorbance at 250 nm of an enzyme solution (0.30 mg/ml in 20 mM acetate buffer pH 6.0) and deithylpyrocarbonate treated enzyme was measured in an Aminco-Chance DW2 spectrophotometer using cells of 1-cm light path. Equal volumes (25 μl) of suitably diluted diethylpyrocarbonate and ethanol were added to the sample and reference cuvettes (1 ml of enzyme solution in each), respectively. The absorbance change at 250 nm was measured since this is close to the trough in the enzyme's absorbance spectrum. The extinction coefficient of carbethoxyhistidine at this wavelength is 1900 $l \cdot mol^{-1} \cdot cm^{-1}$ [36]. The absorbance at 280 nm was also monitored. Corresponding observations were made of the enzyme activity under the same conditions.

Reversal of diethylpyrocarbonate inhibition by hydroxylamine

Aliquots (0.5 ml) of enzyme solution (0.04 mg/ml in 20 mM acetate pH 6) were incubated with 12.5 μl of appropriately diluted diethylpyrocarbonate for 20 min at 30°C. Aliquots of hydroxylamine (0.5 ml of 2 M hydroxylamine in buffer 2 i.e. 0.2 M phosphate buffer containing 1 mM EDTA adjusted to pH 7.0 with H_3PO_4) were added and incubated for 15 min at 30°C. The pH of these samples was promptly reduced to 6 with 1 M acetic acid and the samples dialysed against 2 × 400 vols. of 0.3 M acetate pH 4.0 for 4 h at 4°C. The activities were then assayed and corrected for the minor dialysis dilutions (about 15%) incurred. The polygalacturonase activity was labile at pH 7.0 and a control treatment consisted of the procedure described above using buffer 2 without hydroxylamine. The enzymes were quite stable at pH 6.0 (less than 10% of the activity lost after 4 h at 20°C).

Results

A dialysed solution of Pectinol is distilled water (13 mg/ml) had polygalacturonase, pectin lyase and pectin esterase activities of 4.1, 0.09 and 0.11 units/mg, respectively. No pectate lyase activity was detectable. The pH optimum of the pectin esterase was 4.5 while that of the pectin lyase was 6, similar to several other fungal pectin lyases [2]. The polygalacturonase activity was very specific for polygalacturonic acid substrate rather than pectin. The initial rates were 4.1 and 0.8 units/mg, respectively. Reducing group release continued until the equivalent of 76% of the glycosidic bonds in polygalacturonic acid but only 10% of the pectin bonds were hydrolysed. This is similar specificity to that reported for *Fusarium oxysporum* [14] and *Verticillium albo-atrum* [22] polygalacturonases. The polygalacturonase activity was not affected by iodoacetate (1 mM) or EDTA (10 mM).

DEAE-cellulose chromatography resolved the polygalacturonase activity into two fractions E_1 and E_2 as shown in Fig. 1. E_2 was eluted at a similar position to pectin lyase by the sodium chloride gradient. Subsequent chromatography on Sephadex G-75 separated these two activities (Fig. 2). The yields and specific activities of E_1 and E_2 are shown in Table I. Pectin esterase activity could not be detected in either of the final E_1 and E_2 concentrates; ie the esterase activity is less than 3% of that in the dialysed Pectinol solution (<0.003 units/mg). The molecular weights of E_1 and E_2 calculated from their elution volumes

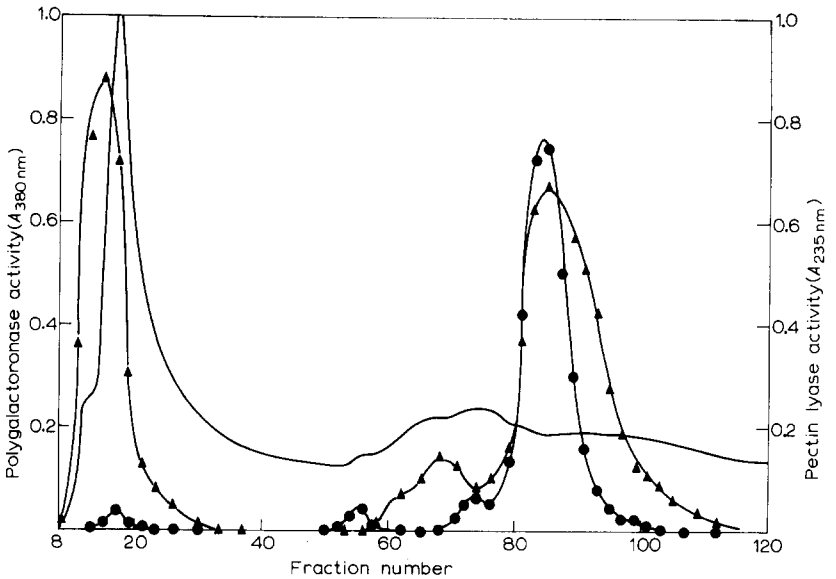

Fig. 1. Elution of Pectinol from DEAE-cellulose by a linear NaCl gradient. A column (15 × 4.5 cm) of DEAE-cellulose was equilibrated in 0.1 M Na_2HPO_4 adjusted to pH 5.2 with 50 mM citric acid (buffer 1). 25 ml of dialysed Pectinol was pumped (175 ml/h) into the column which was then washed with 1 column volume of buffer 1. A linear gradient of 900 ml buffer 1 and 900 ml buffer 1 containing 0.7 M NaCl was used to elute the proteins (started at fraction 23). The fraction size was 14.6 ml. Key to symbols: ———, absorbance at 280 nm; ▲———▲, absorbance at 380 nm (polygalacturonase activity); ●———●, absorbance at 235 nm (pectin lyase activity).

on G-75 (Fig. 2) are 35 000 and >80 000 (close to the G-75 exclusion limit). Chromatography on Sephadex G-150 showed that the molecular weight of E_2 is 85 000. Inclusion of 0.1 M NaCl in the 0.1 M acetate buffer caused the apparent molecular weight of E_2 to decrease to about 42 000 indicating possible

TABLE I

PURIFICATION AND YIELD OF E_1 AND E_2 FROM PECTINOL

Fraction	Total Units	Protein (mg)	Units/mg	Purification	Yield %
Dialysed Pectinol (3 g in 25 ml)	1380	337	4.1	—	100
DEAE-cellulose column					
E_1 after dialysis and freeze-drying	500	41.0	12.2	3	36.2
E_2 after dialysis and freeze-drying	510	18.8	27.1	6.6	37.0
G-75 column					
E_1 after dialysis and freeze-drying	434	5.4	80.8	19.7	31.4
E_2 after dialysis and freeze-drying	350	8.0	43.9	10.7	25.4

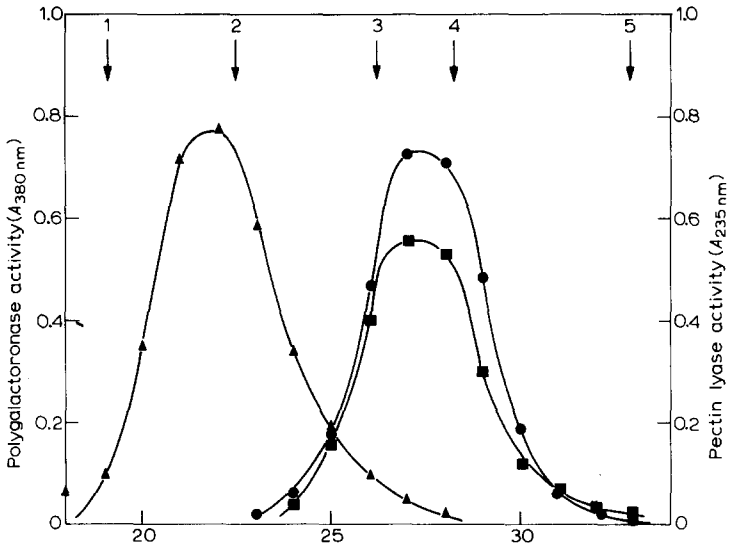

Fig. 2. Resolution of E_2 and pectin lyase on Sephadex G-75. A 6 ml sample of E_2 prepared as described in Materials and Methods was applied to a column (57 × 2.7 cm) of Sephadex G-75 equilibrated in 0.1 M acetate buffer, pH 4.0. The flow rate was 44.7 ml/h and 6.4 ml fractions were collected. A 6 ml sample of E_1 was subsequently applied to the column. Key to symbols: ▲———▲, E_2 (absorbance at 380 nm); ●———●, pectin lyase (absorbance at 235 nm); ■———■, E_1 (absorbance at 380 nm). The elution positions of the following mol.wt. markers are also shown: 1. Dextran Blue. 2. Bovine serum albumin (67 000). 3. Chicken albumin (45 000). 4. Pepsin (34 000). 5. Cytochrome *c* (12 270).

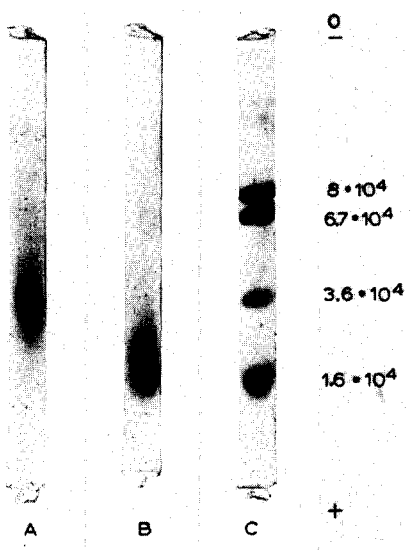

Plate 1. Polyacrylamide gel electrophoretic analysis of E_1 and E_2. Electrophoresis was done in 5% (w/v) polyacrylamide gels containing 0.1% sodium dodecyl sulphate and Tris/acetate/EDTA buffer consisting of 40 mM Tris base/20 mM sodium acetate/2 mM EDTA, adjusted to pH 7.4 with acetic acid. The gels were run at 5 V/cm for 2.5 h. A: E_2 (Fraction 20 of Fig. 2); B: E_1 (Fraction 27 of Fig. 2); C: reference gel: ovotransferrin, bovine serum albumin, lactate dehydrogenase and myoglobin (mol.wts. indicated).

ionic-strength-dependent enzyme dissociation. Both E_1 and E_2 hydrolysed 7% of the glycosidic bonds of polygalacturonic acid when the viscosity of the substrate was reduced by 50%. This is consistent with them both being endopolygalacturonases [2]. The specific activities of these preparations (Table I) and their pH optima differ; the optimal pH values of E_1 and E_2 being 4.1 and 3.8, respectively. The optimal activities of E_1 and E_2 at pH 4.05 in acetate buffer occur in 0.5 and 0.15 M buffer, respectively. The activity of E_2 is less sensitive to ionic strength than E_1 under these conditions. The activity of E_2 changes by less than 10% of the optimal activity in the range 0.01—0.8 M acetate. The corresponding figure for E_1 is about 55%.

The electrophoretic mobilities of E_1 and E_2 on sodium dodecyl sulphate polyacrylamide gels are markedly different. Both bands are diffuse (Plate 1) suggesting heterogeneity. This diffuseness was not improved by different electrophoretic conditions. The approximate molecular weights of E_1 and E_2 are 20 000 and 35 000 respectively with a range of about 6000 (6 measurements). These molecular weights are about half those obtained by gel filtration (Fig. 2). The different mobilities are not dependent on disulphide bonds since the samples were prepared in 1% 2-mercaptoethanol [34].

Enzyme inhibition by diethylpyrocarbonate

Both E_1 and E_2 are inhibited at approximately the same rate at the same protein concentration by diethylpyrocarbonate at pH 6.0. E_2 is partially protected from inhibition by inclusion of 1 or 2% substrate in the incubation medium (Fig. 3). No such protection of E_1 was detected. Both enzymes turn over very slowly at pH 6 and endopolygalacturonase activity from *A. niger* has a

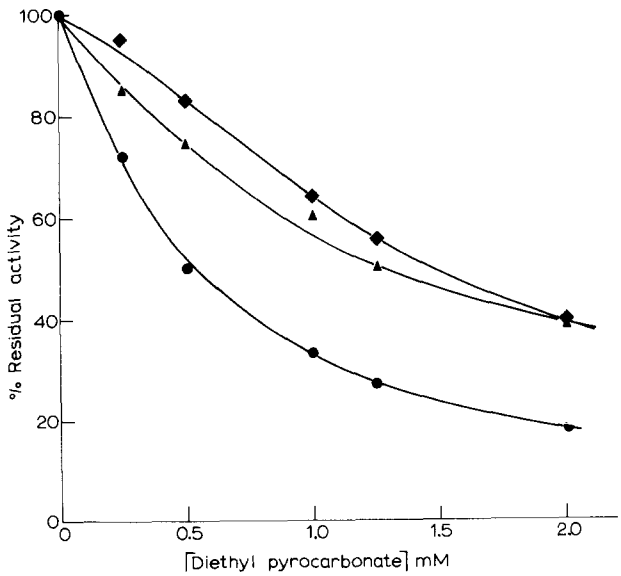

Fig. 3. The inhibition of E_2 by diethylpyrocarbonate. E_2 (0.04 mg/ml in 20 mM acetate pH 6) was incubated with various concentrations of diethylpyrocarbonate for 20 min at 30°C. The residual enzyme activity was then determined (●———●). The incubation was repeated in the presence of 1% (▲———▲) and 2% (◆———◆) polygalacturonic acid at pH 6 and the residual activity determined.

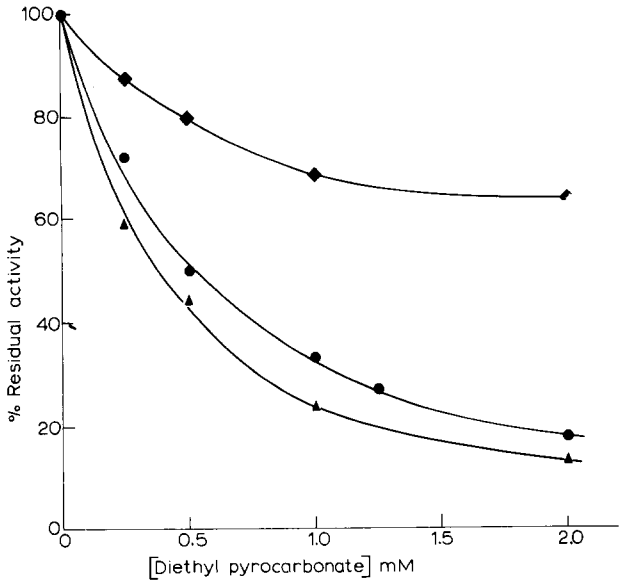

Fig. 4. Reversal of diethylpyrocarbonate inhibition by hydroxylamine. E_2 (0.04 mg/ml in 20 mM acetate, pH 6) was incubated with various concentrations of inhibitor for 20 min at 30°C, and the residual activity determined (●———●). Aliquots of neutral hydroxylamine in buffer 2 were added, mixed and incubated for 15 min at 30°C. The pH of the solutions was then reduced to 6 and the samples dialysed (Materials and Methods) prior to assay (◆———◆). A control treatment with buffer 2 was also assayed (▲———▲).

weak substrate affinity at pH 6 [37]. This suggests that substrate protection might be more effective at a lower pH.

Isolation of the amino acids modified by diethylpyrocarbonate is not possible because these derivatives are not stable in the conditions required for protein hydrolysis [27]. This reagent reacts readily with amine groups, but reactivation of inhibited enzyme by neutral hydroxylamine (Fig. 4) shows that reaction with amine groups is not the cause of inhibition [27,28]. Polygalacturonase activity was labile at pH 7.0 and the brief exposure to this pH involved in the hydroxylamine treatment caused loss of activity. This is shown by the lower line in Fig. 4, and indicates that the effective enzyme reactivation is greater than that represented by the top line in Fig. 4.

Several authors [36,38–40] have reported that diethylpyrocarbonate shows some specificity for histidine residues in proteins at pH 6. This reaction is accompanied by an increase in protein absorbance at 240 nm. Addition of diethylpyrocarbonate to both E_1 and E_2 at pH 6 caused such an increase (monitored at 250 nm as described in Materials and Methods). The rate of inhibition followed the rate of increase in absorbance closely (Fig. 5). This suggests that the same reaction is being measured, the rate constant of which is $3.2 \cdot 10^{-3}$ s^{-1} for E_2. Formation of carbethoxytyrosine is characterised by a negative difference absorbance at 280 nm [41]. A small, rapid decrease in absorbance at 280 nm (10% of that occurring at 250 nm, and completed in 2 min) was observed and it may be the result of contaminant denatured protein. A similar decrease in absorbance at 280 nm accompanied the inhibition of phospho-

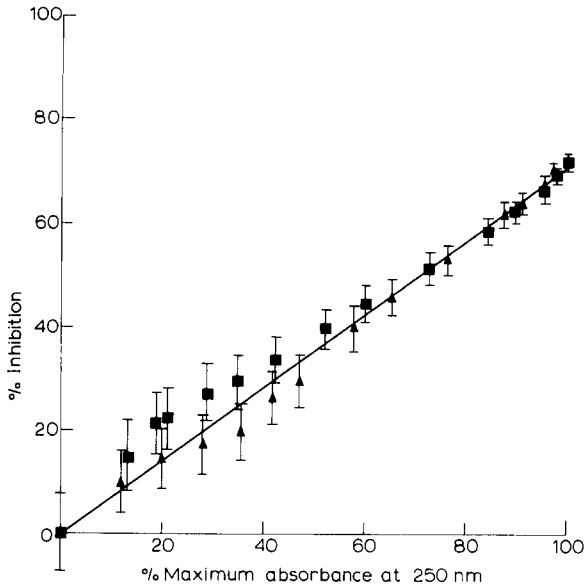

Fig. 5. The change in absorbance at 250 nm accompanying the inhibition of E_1 (■———■) and E_2 (▲———▲) by diethylpyrocarbonate. The enzyme solutions (0.3 mg/ml in 20 mM acetate pH 6) were incubated with inhibitor (1 mM) and the change in absorbance measured (Materials and Methods). The corresponding rate of inhibition was determined and compared with the change in absorbance as shown.

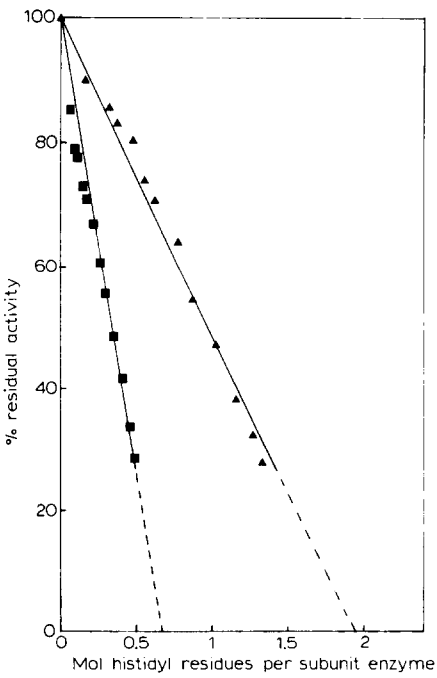

Fig. 6. The relationship between the inhibition of E_1 and E_2 and the number of histidine residues modified. The data in Fig. 5 for E_2 (▲———▲) and the corresponding data for E_1 (■———■) was converted to mol histidine modified per subunit. The subunit weights of E_1 and E_2 were taken as 20 000 and 35 000 respectively.

fructokinase by diethylpyrocarbonate [39]. Labelling studies showed that histidine was the primary group modified [39].

The rate of change of absorbance at 250 nm of E_1 is very similar to that of E_2 (within 10%) and reflected the rate of inhibition. However, the amplitude of the absorbance change was only 60% of that for E_2 at the same protein concentration. The stoicheiometry of carbethoxyhistidine formation for E_1 and E_2 is shown in Fig. 6. The subunit weights used (20 000 and 35 000 for E_1 and E_2) were based on the gel electrophoretic bands which were difficult to define and therefore inaccurate (the stoicheiometries are not greatly different if the monomer weights calculated from gel filtration, i.e. 17 500 and 42 000 are used). The calculated molarities may also be inaccurate because of the assumptions involved in the protein measurement [30]. Fig. 6 shows that about 2 molecules of histidine are modified per subunit of E_2, whereas only 0.6 mol/subunit of E_1 are modified.

Discussion

The two endopolygalacturonases isolated in this study have different molecular and subunit weights, different pH and ionic strength optima and different specific activities. They also differ with regard to substrate protection from diethylpyrocarbonate and the number of reactive histidine residues per molecule. Koller and Neukom [9] separated 3 endopolygalacturonases from another commercial preparation, Pektinex, by cellulose-phosphate chromatography. These enzymes had different pH optima and different activities on oligosaccharides. Two endopolygalacturonases have been prepared from *Rhizopus tritici* by cellulose phosphate chromatography [16] but no information about the two enzymes was presented. The polygalacturonase activity of *Botrytis cinerea* has been resolved into two components, but only one has been characterised [15]. Three *Coniothyrium diplodiella* endopolygalacturonases exist which differ with regard to their specific activities and molecular weights [10—12].

Different forms of polygalacturonase have been prepared from *Penicillium expansum* [13] and *Fusarium oxysporum* f.sp. lycopersici [14]. The enzyme from *P. expansum* was observed to change properties during purification; this was attributed to changes in molecular configuration. The *F. oxysporum* enzyme changed its affinity for DEAE-cellulose during purification on CM-cellulose and hydroxyapatite. This was interpreted as a change in molecular charge and attributed to dissociation of an enzyme-substrate complex [14]. Such complex formation could readily explain differing molecular weights and specific activities (Fig. 2, Table I) but not the different mobilities on sodium dodecyl sulphate polyacrylamide gels and the different stoicheiometries of histidine modification. The two endopolygalacturonases isolated from *Sclerotina fructigena* have molecular weights of 77 000 and 38 500 [18] suggesting that one may be a dimer of the other. These molecular weights are close to those of E_2 and E_1 (85 000 and 35 000), and E_2 was found to dissociate on raising the molarity of the buffer. However, it was still clearly resolved from E_1 by gel filtration. This dissociation of E_2 may explain the difficulty reported in separating pectin lyase from polygalacturonase [22]. Complexes between pectin ester-

ase and polygalacturonase have been reported [15,17]. Both E_1 and E_2 were free of other pectinolytic activity, indicating that such complexes are not the cause of the different forms.

Sodium dodecyl sulphate polyacrylamide gels of E_1 and E_2 were diffuse (Plate 1). Proteolysis during fractionation may be the explanation for this heterogeneity. This is unlikely to have occurred during preparation of the proteins for electrophoresis since phenylmethanesulphonyl fluoride was added to the gel buffer prior to addition of the samples [42]. However, extracellular fungal preparations often have proteolytic activity [43] and this could result in proteolysis during purification procedures causing multiple forms of the enzyme. This may explain the complexity of some polygalacturonase systems containing from 3 to 6 forms of the enzyme [14] and perhaps the existence of minor polygalacturonase components (Fig. 1).

The endopolygalacturonase from *B. cinerea* is inhibited by *p*-chloromercuribenzoate [15]. Several other fungal polygalacturonases, including those studied in the present work, are not inhibited by compounds that react with sulphydryl groups [18,22—24]. The enzyme from *V. albo-atrum* is inhibited by oxidised phenols [24]. These inhibitors are non-specific and inhibition could have resulted from the modification of histidine or proline as well as lysine residues.

The rate of change of absorbance of E_1 and E_2 accompanying inhibition by diethylpyrocarbonate indicated that histidine residues essential for enzyme activity were modified (Fig. 5). If other amino acids are involved in the inhibition, their rates of modification would have to be very similar to the rate of carbethoxyhistidine formation. The reversibility of the inhibition by hydroxylamine (Fig. 4) showed that lysine modification was not involved. Cysteine modification is also unlikely since iodoacetate did not inhibit the enzyme. Rexova-Benkova and Slezarik [26] studied the rate and pH profile of photoinactivation of *A. niger* endopolygalacturonase. This provided indirect evidence that histidine residues are essential for activity (tryptophan, but not tyrosine, was also destroyed by the photooxidation). These authors found that 5 of the 7 histidines per molecule (mol. wt. 35 000) reacted at a faster rate, and suggested that 1 of these is essential for activity [26]. The present work indicates that the number of essential histidines depends on the form of the endopolygalacturonase studied (Fig. 6).

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References

1 Veldhuis, M.K. (1971) in Fruit and Vegetable Juice Processing Technology (Tressler, D.K. and Joslyn, M.A., eds.), 2nd edn. pp. 31—91, Avi Publishing Co., Westport, Connecticut
2 Rombouts, F.M. and Pilnik, W. (1972) Crit. Rev. Food. Technol. 3, 1—25
3 Baker, R.A. and Bruemmer, J.H. (1972) J. Agric. Food Chem. 20, 1169—1173
4 Krop, J.J.P. and Pilnik, W. (1974) Lebensm.-Wiss. Technol.7, 121—124

5 Ishii, S. and Yokotsuka, T. (1971) J. Agric. Food Chem. 19, 958—961
6 Fogarty, W.M. and Ward, O.P. (1972) Process Biochem. 7 (8) 13—17
7 Strand, L.L. and Mussell, H. (1975) Phytopathology 65, 830—831
8 Cooper, R.M. and Wood, R.K.S. (1973) Nature 246, 309—311
9 Koller, A. and Neukom, H. (1967) Mitt. Geb. Lebensmittelunters. Hyg. 58, 512—517
10 Endo, A. (1964) Agric. Biol. Chem. 28, 535—542
11 Endo, A. (1964) Agric. Biol. Chem. 28, 543—550
12 Endo, A. (1964) Agric. Biol. Chem. 28, 551—558
13 Swinburne, T.R. and Corden, M.E. (1969) J. Gen. Microbiol. 55, 75—87
14 Harman, G.E. and Corden, M.E. (1972) Biochim. Biophys. Acta 264, 328—338
15 Urbanek, H. and Zalewska-Soblzak, J. (1975) Biochim. Biophys. Acta 377, 402—409
16 McClendon, J.H. and Kreisher, J.H. (1963) Anal. Biochem. 5, 295—312
17 Miller, L. and Macmillan, J.D. (1971) Biochemistry 10, 570—576
18 Fielding, A.H. and Byrde, R.J.W. (1969) J. Gen. Microbiol. 58, 73—84
19 Mill, P.J. and Tuttobello, R. (1961) Biochem. J. 79, 57—64
20 Rexova-Benkova, L. and Slezarik, A. (1966) Coll. Czech. Chem. Commun. 31, 122—129
21 Lanzarini, G. and Zamorani, A. (1975) J. Sci. Food Agric. 26, 197—205
22 Wang, M.C. and Keen, N.T. (1970) Arch. Biochem. Biophys. 141, 749—757
23 Nyeste, L. and Hollo, J. and Juhasz, S. (1964) Nahrung 8, 373—382
24 Patil, S.S. and Dimond, A.E. (1967) Phytopathology 57, 492—496
25 Luh, B.S. and Phaff, H.J. (1954) Arch. Biochem. Biophys. 48, 23—37
26 Rexova-Benkova, L. and Slezarik, A. (1970) Coll. Czech. Chem. Commun. 35, 1255—1260
27 Melchior, W.B. and Fahrney, D. (1970) Biochemistry 9, 251—258
28 Cooke, R.D., Kanagasabapathy, L. and Ferber, C.E.M. (1975) Abstract, Communication, Federation of European Biochemical Societies 10th Meeting, Abstr. No. 953
29 Gaines, T.P. (1973) J. Assoc. Off. Anal. Chem. 56, 1419—1424
30 Dawson, R.M.C., Elliott, D.C., Elliott, W.H. and Jones, K.M. (1969) Data for Biochemical Research, 2nd edn., pp. 625—626, Oxford University Press, Oxford
31 McIlvaine, T.C. (1921) J. Biol. Chem. 49, 183—186
32 Albersheim, P. (1966) in Methods in Enzymology (Neufeld, E.F. and Ginsburg, V., eds.), Vol, VIII, pp. 628—635, Academic Press, New York
33 Andrews, P. (1964) Biochem. J. 91, 222—233
34 Cooke, R.D. and Holbrook, J.J. (1974) Biochem. J. 141, 71—78
35 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
36 Holbrook, J.J. and Ingram, V.A. (1973) Biochem. J. 131, 729—738
37 Rexova-Benkova, L. and Tibensky, V. (1972) Biochim. Biophys. Acta 268, 187—193
38 Miles, E.W. and Kumagai, H. (1974) J. Biol. Chem. 249, 2843—2851
39 Setlow, B. and Mansour, T.E. (1970) J. Biol. Chem. 245, 5524—5533
40 Kumagai, H., Utagawa, T. and Yamada, H. (1975) J. Biol. Chem. 250, 1661—1667
41 Muhlrad, A., Hegyi, G., and Toth, G. (1967) Acta Biochem. Biophys. 2, 19—29
42 Bretscher, M.S. (1971) J. Mol. Biol. 58, 775—781
43 Sodek, J. and Hofman, T. (1970) in Methods in Enzymology (Perlmann, G.E. and Lorand, L., eds.), Vol. XIX, pp. 372—397 Academic Press, New York