The Pectic Enzymes of *Aspergillus niger*

A MERCURY-ACTIVATED EXOPOLYGALACTURONASE

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1. An exopolygalacturonase was separated from a mycelial extract of *Aspergillus niger* with a 290-fold purification and a recovery of 8·6%. 2. The enzyme displayed its full activity only in the presence of Hg^{2+} ions; K_A for mercuric chloride was about 6×10^{-8}M. 3. The mercury-activated enzyme progressively removed the terminal galacturonic acid residues from α-(1→4)-linked galacturonide chains and converted digalacturonic acid, trigalacturonic acid, tetragalacturonic acid and pectic acid into galacturonic acid.

Many enzymes are known which hydrolyse the α-(1→4)-D-galacturonide links in pectic substances (polygalacturonide glycanhydrolases; polygalacturonases; EC 3.2.1.15). The production of mixtures of such enzymes by submerged culture of *Aspergillus niger* (Tuttobello & Mill, 1961) and the separation of an endopolygalacturonase from such a mixture (Mill & Tuttobello, 1961) have been described. Two distinct exopolygalacturonases have now also been obtained.

Demain & Phaff (1957) defined an exopolygalacturonase as an enzyme which hydrolysed the terminal glycosidic bonds of a chain of α-(1→4)-linked D-galacturonic acid residues. This paper describes one of these enzymes whose activity is powerfully enhanced by the presence of traces of Hg^{2+} ions.

MATERIALS AND METHODS

Fermentations. The techniques used for the production of mixtures of pectic enzymes by the submerged culture of *A. niger* strain CH have been reported (Tuttobello & Mill, 1961). The medium was modified by omitting the sucrose and increasing the pectin concentration to 5%; this favoured the production of exopolygalacturonase at the expense of endopolygalacturonase.

Special chemicals. The pectin and pectic acid used were obtained as before (Mill & Tuttobello, 1961).

Digalacturonic acid, obtained as described by Phaff & Luh (1952), was purified by recrystallization from water as its dibrucine salt. Solutions of the free acid were prepared as required by shaking weighed quantities of the salt in water with a cationic exchange resin [Amberlite CG-120 (H^+ form)], filtering, washing the resin thoroughly with water and diluting the combined filtrates to a known volume. The free acid had a $-CO_2H/-CHO$ (iodometric) ratio 2·01. Before use as a substrate these solutions were adjusted to the appropriate pH value with NaOH.

Trigalacturonic acid was prepared as described by Ashby, Brooks & Reid (1955) and recrystallized as its tribrucine salt. The free acid prepared from this as above had a $-CO_2H/-CHO$ ratio 2·99.

Small quantities of a presumed tetragalacturonic acid were obtained during the separation of the trigalacturonic acid. After this had been removed from the chromatographic column, prolonged elution with N-formic acid removed a further small peak of galacturonide material. Chromatography of this on paper as described below gave a single spot whose position, compared with the positions of the monomer, dimer and trimer, was consistent with its being the tetramer. Insufficient material was obtained however for further characterization.

Assays of enzyme activity. Digalacturonic acid, which is not hydrolysed by the endopolygalacturonase of *A. niger* (Mill & Tuttobello, 1961), is a convenient substrate for the assay of exopolygalacturonase. The hydrolysis of digalacturonic acid was followed in a solution containing 0·5% of the free anydrous acid, at 30°. The colorimetric copper method of Cook & Phillips (1957) was used to follow the release of galacturonic acid. This method gave only a slight reaction with digalacturonic acid. The colour intensities obtained were measured in a Spekker absorptiometer with filter 606 and the degree of hydrolysis obtained was evaluated by comparison with standard solutions containing digalacturonic acid and galacturonic acid mixed in proportions simulating various degrees of hydrolysis. Metal ions tested for their effect on the enzyme activity did not interfere with the assay at the concentrations used.

For the routine assay of the enzyme it was diluted to a suitable concentration in 0·05M-sodium acetate buffer, pH4·6, containing 0·01mM-$HgCl_2$. Equal volumes of this solution and a 1% solution of digalacturonic acid, previously adjusted to pH4·6, were mixed in a water bath at 30°. After 2min. and 12min. incubation, 0·05ml. samples were withdrawn and mixed with 0·5ml. of the copper reagent

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(Cook & Phillips, 1957). The mixture was heated for 10min. in a boiling-water bath, cooled, and 1ml. of colour reagent and 10ml. of water were added. The rate of hydrolysis of the substrate remained constant until about 50% of it had been hydrolysed. One unit of activity released 1 μequiv. of galacturonyl reducing group in 1min. at 30° under the conditions described.

Hydrolysis of trigalacturonic acid was measured in a similar manner. Trigalacturonic acid gave no colour with the copper reagent. It was assumed that the enzyme preferentially attacked the trigalacturonic acid before attacking the liberated digalacturonic acid; accordingly the assay was calibrated with mixtures of trigalacturonic acid, digalacturonic acid and galacturonic acid, corresponding to varying degrees of hydrolysis of a single bond in each molecule of substrate. If the digalacturonic acid formed were preferentially hydrolysed, errors would be introduced. However, the initial attack must yield digalacturonic acid and galacturonic acid and the concentration of the dimer will at first be very low, so that if the initial rate of hydrolysis is measured it should approximate to the rate of hydrolysis of a single bond in the trigalacturonic acid molecule. Accordingly, in measuring the rate of hydrolysis with this substrate, samples of the digest were taken for assay at 2min. intervals and the initial rate of hydrolysis was determined from these results.

Ultraviolet measurements. The extinctions of enzyme solutions were measured at 260 and 280mμ with a 1cm. light-path. Protein concentrations were calculated as described by Warburg & Christian (1941).

Chromatography of oligouronides. Paper chromatography was performed as described by Mill & Tuttobello (1961).

Cellulose ion-exchangers. Diethylaminoethylcellulose (DEAE-cellulose) was the Whatman product. Cellulose phosphate was prepared as described by Peterson & Sober (1956).

Propanol precipitations. The technique used for propanol precipitation of the enzyme was identical with that described for ethanol precipitations (Mill & Tuttobello, 1961).

Purification of the enzyme

The following description applies to material obtained by culture of the *A. niger* in 50l. of medium.

Fermentation. Fermentation was continued for 100hr. At this stage the enzyme activity remained associated with the mycelium and was released into the medium only if fermentation were prolonged. The mycelium was collected by vacuum filtration with the aid of diatomaceous earth. As much fluid was removed as possible and the filter cake was washed with 5 vol. of ice-cold 0·1M-sodium acetate buffer, pH4·6.

Stage 1. The filter cake was frozen at −20° overnight and was allowed to thaw at room temperature. It was mixed with 1l. of crushed ice and was disrupted in a large blender for 10min. The mass became much more fluid and was filtered through a bed of diatomaceous earth. The filter cake was washed with 500ml. of water and the washings were added to the filtrate, giving a total volume of 4·2l. After standing for 3 days at 4°, a considerable quantity of solid matter was deposited and was removed by filtering.

Stage 2. The pH of the enzyme solution was adjusted to 4·0 with 0·1N-HCl; it was cooled to 0°, and 1·1 vol. (4·6l.) of propan-1-ol (precooled to −20°) was added slowly with constant stirring. After 4hr. the precipitate produced was collected by centrifuging and suspended in 150ml. of ice-cold water. Insoluble matter was removed by centrifuging, and extracted with 100ml. of water. The two aqueous extracts were combined.

Stage 3. Cellulose phosphate (60g.) was added to the enzyme solution and the mixture was adjusted to pH3·3 with 0·1N-HCl. The mixture was stirred for 20min. and then centrifuged, and the residue was washed with 250ml. of water and drained on a Buchner funnel.

The cellulose phosphate was suspended in 90ml. of 0·2M-sodium acetate buffer, pH3·8, and the pH was readjusted to 3·8 with 0·2M-sodium acetate solution. After stirring for 20min., the cellulose phosphate was collected by centrifuging. The elution was repeated three times and the residue was then washed with 1l. of water.

The enzyme was released from the cellulose phosphate by extracting it four times with 100ml. portions of 0·2M-sodium acetate buffer, pH4·6, each for 20min. at 4°. The pooled eluates were frozen at −20°, thawed at 4° and the precipitated matter was removed by centrifuging.

Stage 4. The enzyme was purified further by chromatography on DEAE-cellulose. The available enzyme was divided into two batches, each of which was treated identically.

A column (15cm.×3·6cm.) of DEAE-cellulose was prepared in 0·2M-sodium acetate buffer, pH4·6. The enzyme solution was dialysed exhaustively against the same buffer and was then percolated through the column. Elution was carried out with sodium acetate buffer, pH4·6, a linear gradient of concentration between 0·02M and 0·4M being used; 1l. of each of these buffers was used. Fractions (about 15ml.) were collected at 10min. intervals. Their extinction at 280mμ was measured. Their ability to hydrolyse digalacturonic acid at pH4·6 in the presence and in the absence of 10 μM-$HgCl_2$ was determined.

In a typical run (Fig. 1) the hydrolytic activity fell into two peaks. The activity in the first peak was enhanced in

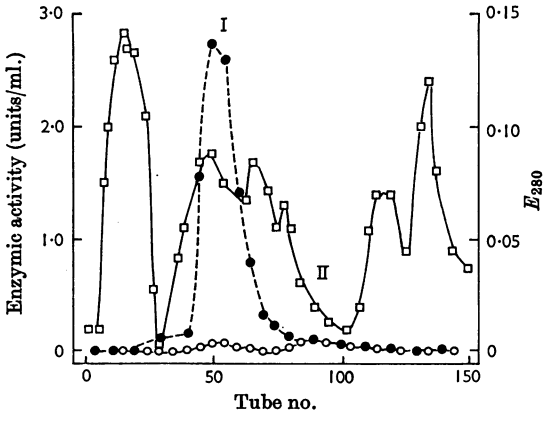

Fig. 1. Chromatography of enzyme preparation (from stage 3) on DEAE-cellulose. For conditions see enzyme purification 'Stage 4'. □, E_{280}; ●, activity in the presence of 10 μM-$HgCl_2$; ○, activity without added $HgCl_2$. Peak I, tubes 42–65; peak II, tubes 82–110.

the presence of $HgCl_2$, whereas that in the second was not. The two peaks were pooled separately. The further investigation of the material in the second peak is considered in the next paper (Mill, 1966).

Stage 5. The pooled material from peak I was dialysed against 6l. of distilled water. A portion (8ml.) of 2M-sodium acetate buffer, pH4·6, was added to the solution of non-diffusible material, and the mixture was percolated through a column (7cm.×1cm.) of DEAE-cellulose in 0·02M-sodium acetate buffer, pH4·6. The enzyme was eluted by washing the column with six 15ml. portions of 0·5M-sodium acetate buffer, pH4·6. The eluates were pooled and dialysed against 2l. of water. This treatment served simply to concentrate the enzyme.

2M-Sodium acetate buffer, pH4·6 (1ml.), was added to the dialysed solution and the mixture was percolated through a column (20cm.×2cm.) of DEAE-cellulose in 0·02M-sodium acetate buffer, pH4·6. The column was eluted with a linear gradient of buffer: the mixing vessel containing 350ml. of 0·05M-sodium acetate buffer, pH4·6, and the reservoir 350ml. of 0·05M-sodium acetate buffer, pH3·6. Fractions (5ml.) were collected at 10min. intervals and assayed for hydrolytic activity against digalacturonic acid at pH4·6 in the presence of 10μM-$HgCl_2$ as before.

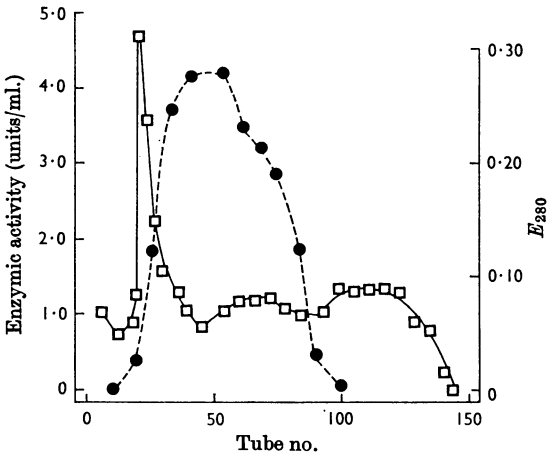

Fig. 2. Chromatography of enzyme preparation (from stage 4, peak I) on DEAE-cellulose. For conditions see enzyme purification 'Stage 5'. □, E_{280}; ●, activity in the presence of 10μM-$HgCl_2$. Peak, tubes 30–75.

An irregular peak of activity was obtained (Fig. 2) and the material from the centre of this peak (tubes 30–75 in the example shown) was collected. The pooled solution was brought to pH4·6 and dialysed against distilled water. Finally this material was concentrated by passing it through a column (2cm.×1cm.) of DEAE-cellulose in 0·02M-sodium acetate buffer, pH4·6, and then eluting it with five 5ml. portions of 0·5M-sodium acetate buffer, pH4·6.

Table 1 summarizes a typical purification procedure. The final preparation contained about 15mg. of protein.

RESULTS

Effect of mercuric ions on the activity of the enzyme. Suitable dilutions of the enzyme were prepared in 0·05M-sodium acetate buffers, pH4·6, containing various amounts of mercuric chloride. The enzyme solutions were held for 5min. at 30° and then tested for their ability to hydrolyse digalacturonic acid. Fig. 3 shows that the enzyme has some

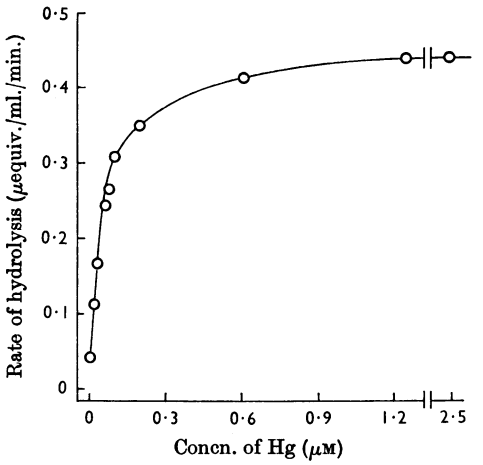

Fig. 3. Effect of mercury on exopolygalacturonase activity. Rates of hydrolysis are shown of 0·5% solutions of digalacturonic acid, at pH4·6 and 30°, by a single preparation of the enzyme in the presence of different concentrations of $HgCl_2$. In each case the enzyme was present at a concentration of 0·44 unit/ml.

Table 1. *Purification of the mercury-activated endopolygalacturonase from a 50l. fermentation*

Fraction	Specific activity (units/mg. of protein)	Relative activity	10^{-3}× Total activity (units)	Percentage of original activity
Stage 1: Mycelial extract	0·24	1	11·6	100
Stage 2: Propanol precipitate	2·10	8·5	11·5	99
Stage 3: Eluate from cellulose phosphate	9·70	40	3·9	34
Stage 4: Pool I from DEAE-cellulose column	29·0	120	1·3	11
Stage 5: Pool from second DEAE-cellulose column	70·0	290	1·0	8·6

activity in the absence of added mercuric chloride but that the addition of this leads to an increase in activity. The results indicate K_A $6{\cdot}2 \times 10^{-8}$M for mercuric chloride.

Action of other agents on the enzyme. Ca^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, Ba^{2+}, Sr^{2+}, Fe^{3+}, Cd^{2+}, Pb^{2+}, Mn^{2+} and Cl^- had no effect on the enzyme when present at 0·1mM. Hg^+ produced an activation although it was not as effective as Hg^{2+}. EDTA (disodium salt) (0·1%) prevented the action of mercuric chloride. Dialysis of a mercury-activated preparation against a saturated aqueous solution of 2,3-dimercaptopropanol followed by dialysis against water led to an almost total loss of activity.

Effect of pH *on the rate of hydrolysis.* Samples of the enzyme solution were diluted to the same concentration in sodium acetate or potassium phthalate buffers of various pH values and were then mixed with neutralized solutions of digalacturonic acid, and the rates of hydrolysis were determined. Finally the pH values of the reaction mixtures were measured. Optimum activity was obtained at pH4·4–4·6 (Fig. 4) with a rapid fall in activity at higher pH values.

Action of the enzyme on different substrates. The rate of hydrolysis induced by a single preparation of the enzyme in the presence of 10μM-mercuric chloride, with digalacturonic acid, trigalacturonic acid, pectic acid, and pectin as substrates, was measured. The breakdown of pectin and pectic acid was followed iodometrically (Mill & Tuttobello, 1961). Digalacturonic acid and trigalacturonic acid were hydrolysed much more rapidly than pectic acid and pectin was virtually resistant to attack (48·5, 41·5, 0·6 and 0·007μequiv. of reducing group/mg. of protein/min. respectively).

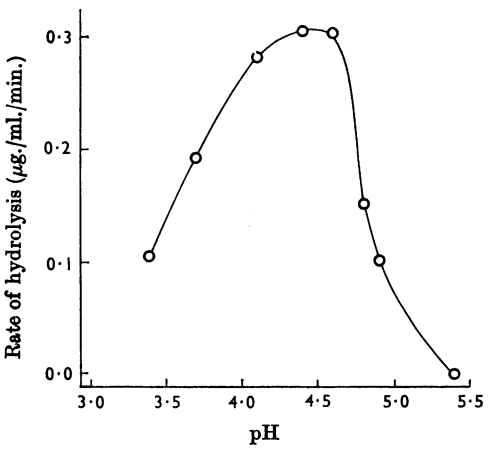

Fig. 4. Effect of pH on enzyme activity. Rates of hydrolysis are shown of 0·5% solutions of digalacturonic acid at 30° in mixtures of various pH values in the presence of 10μM-$HgCl_2$ and 0·31 unit of enzyme activity/ml.

Prolonged digestion of pectic acid resulted in its complete hydrolysis. A 1% solution of pectic acid, brought to pH4·6, was mixed with an equal volume of a solution, containing 12·5units of enzyme activity/ml., in 0·05M-sodium acetate buffer, pH4·6, containing 10μM-mercuric chloride. A few drops of toluene were added and the mixture was incubated at 30°. Samples (2ml.) were assayed at intervals for their content of reducing groups. After 70hr. all the potential reducing groups of the pectic acid had been released.

The hydrolysis of various substrates was also followed chromatographically. As described by Mill & Tuttobello (1961), a 1·0% solution of the substrate at pH4·6 was mixed with a suitable dilution of the enzyme containing 20μM-mercuric chloride. Samples of the reaction mixture were withdrawn at intervals and spotted on to chromatographic paper. The spots were dried at once in a stream of air, and the papers were later developed chromatographically. With digalacturonic acid as substrate a spot appeared corresponding to galacturonic acid. This increased in intensity and the original spot corresponding to digalacturonic acid decreased in intensity, as the reaction proceeded. With trigalacturonic acid, spots corresponding to digalacturonic acid and galacturonic acid appeared at the same time. The latter steadily increased in intensity but the former remained fairly constant until the original spot corresponding to trigalacturonic acid had almost disappeared. The digalacturonic acid spot then faded also.

Similarly with tetragalacturonic acid, the first new spots to appear corresponded to trigalacturonic acid and galacturonic acid. These were followed by a faint spot corresponding to digalacturonic acid. As the reaction reached completion the oligomer spots disappeared in the inverse order to their appearance, i.e. first tetragalacturonic acid, then trigalacturonic acid, then the digalacturonic acid. The spot corresponding to the galacturonic acid increased throughout.

When pectic acid was similarly treated only galacturonic acid could be detected; even after 48hr. hydrolysis no higher oligomer could be seen.

Stability of the enzyme. The enzyme was characterized by a low stability in neutral solution and a relatively high stability in acid solution. A sample of the enzyme heated for 10min. at 50° in a solution of pH7·0 and then readjusted to pH4·6 had only 2% of its original activity, whereas after heating at 50° for 1hr. in a solution of pH2·5 the enzyme retained 18% of its activity when readjusted to pH4·6. The presence or absence of the 10μM-mercuric chloride had no effect on the stability of the enzyme.

DISCUSSION

The existence of exopolygalacturonases, i.e. enzymes which attack polygalacturonide chains by removing the terminal galacturonide residue, is well documented (Ayres, Dingle, Phipps, Reid & Solomons, 1952; Dingle, Reid & Solomons, 1953; Saito, 1955).

The chromatographic study of the products produced by the action of the mercury-activated enzyme on pectic acid and the oligouronides makes it clear that this enzyme is also removing terminal galacturonide molecules. The hydrolysis of the digalacturonic acid is proof of the ability of the enzyme to hydrolyse such bonds; the breakdown of the tetragalacturonic acid first produced trigalacturonic acid and galacturonic acid, indicating that the tetramer suffered a 3 + 1 split; the detection of galacturonic acid as the sole product of the breakdown of pectic acid shows that here, too, the enzyme was acting by liberating terminal galacturonic acids. On prolonged hydrolysis, each of the original pectic acid chains should give rise to a molecule of oligomer which would progressively decrease in length, but because of the high molecular weight of pectic acid the numbers of such oligomer molecules would be relatively small and they would not be easily detected.

The enzyme had only a very feeble action against pectin, showing that it was a polygalacturonase rather than a polymethylgalacturonase. The low activity against pectic acid compared with that against digalacturonic acid may well reflect not a difference in susceptibility of the terminal bonds in the two substrates, but simply the difference in the effective concentrations of the terminal bonds: the low molecular weight dimer has many more terminal bonds than the polymer when both substrates are at the same concentration.

The interesting feature of this enzyme is undoubtedly its activation by mercury ions. No other ions could be found which were active in this system and the effect was not due to the chloride ions. It seems probable that the enzyme would be completely inactive if mercury could be completely removed. The feeble activity of the preparation in the absence of mercury could be accounted for by the chance presence of mercury at a concentration of less than 10^{-8}M. Dialysis against a solution of 2,3-dimercaptopropanol and then against water removed even this feeble activity. It is, however, difficult to visualize the role of the enzyme *in vivo* for it would not generally be supposed that the mycelium contained even a very low concentration of mercury.

Possibly the enzyme is constrained *in vivo* into a particular configuration by structural features of the cell or by a chemical combination. Extraction of the enzyme might then release it from this configuration and so destroy the activity, which could be restored when the enzyme regained its configuration by combining with the mercury, e.g. by a disulphide–mercury bridge. If this were so then the situation would differ from that with normal metal-activated enzymes, for under conditions *in vivo* the mercury would play no part in the reaction. Moreover, the mercury would not necessarily be involved at the catalytic site of the enzyme.

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