The Pectic Enzymes of *Aspergillus niger*

A SECOND EXOPOLYGALACTURONASE

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1. A second exopolygalacturonase was separated from a mycelial extract of *Aspergillus niger* with a 265-fold purification and a recovery of 1%. 2. Unlike the first exopolygalacturonase this enzyme showed no requirement for metal activators, nor was it inhibited by chelating agents. 3. The two exopolygalacturonases were also distinguished by their pH optima and stability. 4. The enzyme progressively removed the terminal galacturonic residues from α-(1→4)-linked galacturonide chains, converting digalacturonic acid, trigalacturonic acid and tetragalacturonic acid into galacturonic acid. Galacturonic acid was also released from pectic acid but complete digestion was not achieved.

The preparation and characterization of two pectolytic enzymes from a single strain of *Aspergillus niger* have been described (Tuttobello & Mill, 1961; Mill & Tuttobello, 1961; Mill, 1966). One of these enzymes was an endopolygalacturonase which hydrolysed polygalacturonic acid chains by a random mechanism of attack. The other enzyme was an exopolygalacturonase which hydrolysed such chains by a terminal mechanism of attack. This latter enzyme was activated by mercury ions, but during its preparation evidence was obtained of a second exopolygalacturonase which did not require such activation. This paper describes the further characterization of this enzyme.

MATERIALS AND METHODS

The materials and techniques used in the investigation of this enzyme have been described (Mill, 1966). The enzyme was assayed as described for the mercury-activated enzyme but was diluted in 0·1M-sodium acetate buffer, pH5·0, containing 0·1% of EDTA (disodium salt). The digestion period was extended to 30min. because of the low activities encountered.

Preparation of the enzyme. The early stages of the preparation of this enzyme are the same as those for the preparation of the mercury-activated enzyme, but at stage 4 in that preparation (Mill, 1966), two peaks of exopolygalacturonase activity were obtained. The enzyme forming the second peak (II) was not affected by low concentrations of mercuric chloride. The pooled material from this peak formed the starting material for the present investigation.

Stage 5a. The pooled material from the second peak 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 procedure served simply to concentrate the enzyme.

This material was percolated through a column (20cm. ×1·2cm.) of DEAE-cellulose prepared in 0·02M-sodium acetate buffer, pH4·6. The column was eluted with a linear gradient between 350ml. of 0·12M-sodium acetate

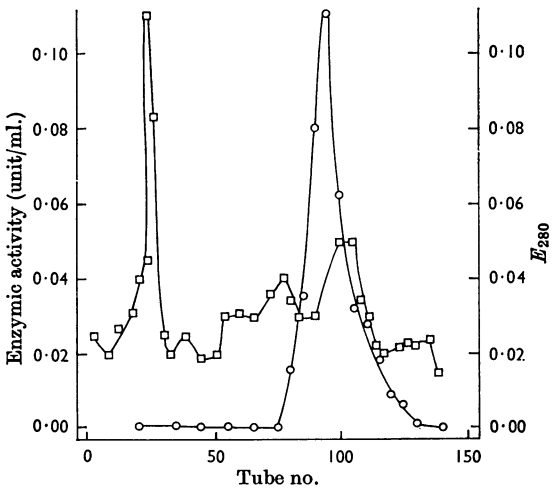

Fig. 1. Chromatography of the exopolygalacturonase preparation on DEAE-cellulose. For conditions see text. □, E_{280}; ○, activity. Peak, tubes 80–120.

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Table 1. *Purification of exopolygalacturonase from a 50l. fermentation*

Fraction	Specific activity (units/mg. of protein)	Relative activity	Total activity (units)	Percentage of original activity
Stage 1: Mycelial extract	0·029	1	1400	100
Stage 2: Propanol precipitate	0·20	7	1100	78
Stage 3: Eluate from cellulose phosphate	1·1	38	500	36
Stage 4: Pool II from DEAE-cellulose column	4·2	145	44	3
Stage 5: Pool from second DEAE-cellulose column	7·7	265	12	1

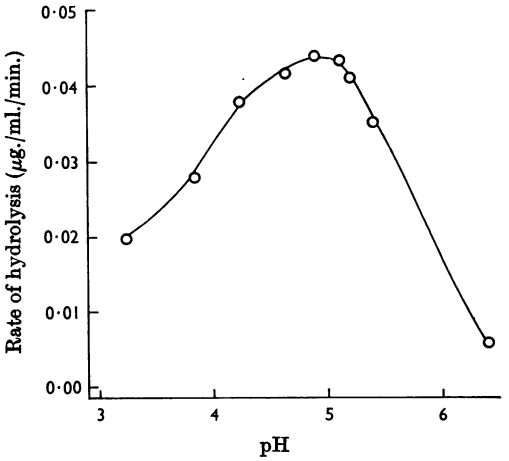

Fig. 2. Effect of pH on enzyme activity. Rates of hydrolysis for 0·5% solutions of digalacturonic acid at 30° in mixtures of various pH values in the presence of 0·044 unit of enzyme activity/ml. are shown.

buffer, pH 4·6, in a mixing vessel and 350ml. of 0·15M-acetic acid in a reservoir. Fractions (5ml.) were collected. The extinction of the fractions was measured at 280mμ and they were assayed for their ability to hydrolyse digalacturonic acid (Fig. 1). A single peak of activity was obtained.

The fractions containing the activity were pooled and dialysed against 5l. of water. The pH was adjusted to 4·8 and the enzyme solution was percolated through a column (2cm. × 1cm.) of DEAE-cellulose in 0·05M-sodium acetate buffer, pH 4·6. The column was eluted with three 5ml. portions of 0·5M-sodium acetate buffer, pH 4·6. The combined eluates were dialysed against water. Table 1 summarizes the results of such a purification. It is possible that the assays for stages 1, 2 and 3 are too high; the mercury-activated enzyme may be contributing to the results.

RESULTS

Effect of mercury ions. The rate at which the stage 5a preparation hydrolysed digalacturonic acid was unaffected by the addition, to the reaction mixture, of mercuric chloride in a final concentration of 10μM.

Table 2. *Comparison of the rates of hydrolysis of 0·5% solutions of various substrates by a single enzyme preparation at* pH 5·1 *and* 30°

Substrate	Reducing groups liberated (μequiv./ml. of enzyme protein/min.)
Digalacturonic acid	4·9
Trigalacturonic acid	6·3
Pectic acid	0·47
Pectin	0·00

Effect of chelating agents. The addition of 0·1% EDTA (disodium salt), or dialysis against a saturated solution of 2,3-dimercaptopropanol and then against water, did not alter the rate of the enzymic reaction.

Effect of pH *on the rate of hydrolysis.* Samples of the enzyme solution were diluted to the same extent in 0·1M-sodium acetate or potassium phthalate–hydrochloric acid buffers of various pH values, and were then mixed with equal volumes of a neutralized 1·0% solution of digalacturonic acid at 30°. The rate of hydrolysis was determined and the pH values of the enzymic digests were measured. Optimum activity was found at pH 5·0–5·1 (Fig. 2).

Action of the enzyme on different substrates. Table 2 shows the rate of hydrolysis induced by a single preparation of the enzyme at pH 5·1, with digalacturonic acid, trigalacturonic acid, pectic acid, and pectin. The digalacturonic acid and trigalacturonic acid were attacked much more rapidly than pectic acid, and pectin was resistant to attack. The hydrolysis of various substrates was also followed chromatographically (Mill & Tuttobello, 1961). In each case 0·5% solutions of the substrate were digested at 30° in 0·05M-sodium acetate buffer, pH 5·0. Samples of the digests were taken at intervals, spotted on to the chromatographic paper, and dried in a stream of air. Development of the chromatogram revealed the manner in

which the substrate was broken down to the galacturonide units.

With digalacturonic acid as substrate, galacturonic acid was rapidly produced and increased in amount and the digalacturonic acid decreased and finally disappeared. With trigalacturonic acid as substrate, both digalacturonic acid and galacturonic acid first appeared. The galacturonic acid steadily increased but the digalacturonic acid showed only a slight increase and finally both the trigalacturonic acid and the digalacturonic acid disappeared.

With pectic acid as substrate only galacturonic acid was detected as a product.

When the total extent of hydrolysis of pectic acid by the enzyme was followed, it was found that even after 8hr. incubation at 30° only 28% of the total reducing groups of the pectic acid had been liberated. It is not clear whether this reflects an inherent inability of the enzyme to attack the substrate or the relatively low activity of the enzyme.

Stability of the enzyme. A sample of the enzyme heated for 10min. at 50° in solution at pH 7 retained 48·5% of its activity. When heated at 50° at pH 2·5 it retained 4·5% of its activity after 1hr.

DISCUSSION

The chromatographic study of the reaction products from the hydrolysis of the various substrates shows that this enzyme is, like the mercury-activated enzyme, an exopolygalacturonase. Although it was not possible to digest pectic acid completely with this enzyme, this might have been due to its relatively low activity, since the oligomers were completely hydrolysed. Once again the low activity against pectic acid compared with that against the oligomers may be due to the low effective concentration of susceptible terminal bonds present at any time in the polymer.

The question arises of the relationship of this enzyme to the first, mercury-activated, enzyme (Mill, 1966). Both have the same pattern of catalytic activity but differ in a number of ways.

1. Mercury greatly activates the first enzyme but not the second.

2. The enzymes can be separated on ion-exchange celluloses.

3. The mercury-activated enzyme has a pH optimum of 4·6 and shows a rapid fall in activity if the pH is raised; the second enzyme has optimum pH 5·0–5·1.

4. The mercury-activated enzyme is slightly less stable than the other at pH 7 but more stable at pH 2·5.

These considerations make it clear that the two enzymes are present in the preparation as different forms. Nor can it be assumed that the second enzyme represents the first enzyme already containing mercury. If this were the case the pH optima should be the same, the stability of the mercury-activated enzyme in the presence of mercury should be the same as that of the second enzyme, and it is not, and the second enzyme should show some inhibition in the presence of chelating agents, and it does not. There remain two possibilities. First, the mercury-activated enzyme may represent a chemical modification of the second enzyme, e.g. a disulphide bridge in the second enzyme might become oxidized with a loss in enzyme activity which might be restored by forming a mercury–disulphide bridge. Secondly, the two enzymes might indeed be distinct.

If the relative rates of hydrolysis (Table 3) of the digalacturonic acid, trigalacturonic acid and pectic acid by the endopolygalacturonase (Mill & Tuttobello, 1961) and the two exopolygalacturonases are compared the different patterns of activity of the three enzymes for substrates of different sizes are clearly shown.

Exopolygalacturonases were obtained, free from

Table 3. *Relative rates of hydrolysis of* 0·5% *solutions of digalacturonic acid, trigalacturonic acid and pectic acid by the endopolygalacturonase, the mercury-activated exopolygalacturonase and the second exopolygalacturonase*

For each enzyme the rates are expressed relative to the rate with trigalacturonic acid given as unity.

	Relative rates of release of reducing groups		
	Endopoly-galacturonase	Hg-activated exopoly-galacturonase	Second exopoly-galacturonase
Digalacturonic acid	0	1·2	0·8
Trigalacturonic acid	1	1	1
Pectic acid	960	0·014	0·07

endopolygalacturonase activity, from mycelia of *A. foetidus* (Brooks & Reid, 1955) and *A. niger* (Dingle, Reid & Solomons, 1953; Saito, 1955) and Saito (1955) showed that the enzyme attacked pectin by removing the terminal galacturonic acid residue. Brooks & Reid (1955) reported that a preparation of the *A. foetidus* enzyme hydrolysed digalacturonic acid and trigalacturonic acid.

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