Purification and Properties of Endopolygalacturonase from *Ganoderma lucidum*

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SUMMARY

The enzyme endopolygalacturonase secreted by the fungus *Ganoderma lucidum* was purified by a three-step procedure until pectin methylesterase was no longer detectable. The endopolygalacturonase activity was inhibited by divalent metal ions. The enzyme had a peak of activity at pH 5.4 and two temperature optima: one at 50° and the other at 70°.

INTRODUCTION

The arecanut palms in Mysore State (India) are subject to a disease, 'Anabe roga', or foot rot, caused by the fungus *Ganoderma lucidum*. Although the pathogenicity of the fungus has been recorded (Watt, 1901; Venkatarayan, 1936; Naidu, Kumar & Sannamarappa, 1966), little is known about the physiological and biochemical aspects of this disease.

In many plant diseases the extracellular enzymes and, in particular, pectolytic enzymes secreted by the pathogens are known to play an important role in pathogenesis (Wood, 1959, 1960; Brown, 1955, 1965; Sadasivan & Subramanian, 1963; Bateman & Millar, 1966). Such an enzyme may be active in the formation of rot induced by *Ganoderma lucidum*. The purification and properties of the enzyme endopolygalacturonase (EC 3.2.1.15) are reported in this paper.

METHODS

Chemicals. These were obtained as follows: pectin and polygalacturonic acid from Eastman Kodak Company, galacturonic acid from British Drug Houses and the fungicides maneb, zineb, and Blitox-50 as gifts from Tata Fison Industries Ltd, Bangalore. Other chemicals used were of Analar and reagent grade.

Culture. The culture of *Ganoderma lucidum* was obtained from the Indian Agricultural Research Institute, Delhi, India, and was maintained on potato-dextrose agar (PDA) slants at room temperature. For the preparation of inoculum, a uniform suspension of a 5 to 6 day culture grown on a PDA slant was made. To the culture 5 ml. of sterile distilled water was added. With the inoculating loop, the soft growth on the slant was scraped gently and broken down to small pieces by rubbing against the sides of the test tube. The culture in suspension was transferred to another sterile test tube and shaken with glass beads. From this 0.1 ml. was added to 250 ml. (in a 1 l. Roux flask) of medium which comprised (% w/v): pectin, 1.0%; ammonium

tartrate, 1.0%; KH_2PO_4 , 0.1%; and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05%; initial pH of the medium, 5.0 to 5.2. The culture was incubated at room temperature (22° to 27°).

Assay method. One unit (u) of polygalacturonase (PG) was the amount of enzyme at pH 5.4 and 30° that released 1 μmole of reducing group/min. from polygalacturonic acid. The substrate concentration was 0.5% (w/v) in 0.1 M-sodium acetate buffer. The increase in aldehyde groups was measured in 5 ml. samples of the reaction mixture by hypiodite oxidation (Jansen & MacDonnell, 1945).

The pectin methylesterase (PME) activity was followed by titrating at appropriate intervals the liberated carboxyl groups with 0.02 N-NaOH (Bilimoria, 1961, 1962). The unit of pectin methyl esterase is the amount of enzyme that releases 1 mole of carboxyl groups/min. at pH 6.8 from pectin solution at 30°.

The assays for pectin *trans*-eliminases were based on the enzymic production of materials that have an extinction maximum at 235 nm. (Edstrom & Phaff, 1964). Pectin and polygalacturonic acids were used as substrates.

Protein concentrations were determined by the method of Warburg & Christian (1941).

Paper chromatography. Unidimensional descending paper chromatography on Whatman no. 1 filter paper was carried out with butan-1-ol + acetic acid + water (4:3:3) as solvent system. Fifty to 100 $\mu\text{l.}$ of the reaction mixture was applied to the filter paper. The strips were developed at room temperature for 18 h. Dried papers were sprayed with a solution of 0.1% (w/v) bromophenol blue in 95% aqueous ethanol (pH 6.5). The galacturonic acid was identified by comparison with an authentic sample. The di-, tri- and tetragalacturonides were identified by calculation of R_m value and plotting a graph, as shown by Hathway & Seakins (1958), where

$$R_m = \log (1/R_f - 1).$$

RESULTS

An initial test of the culture fluid and a cell-free extract of the mycelium showed the presence of polygalacturonase and pectin methylesterase activities. However, the PG and PME activities were higher in the culture fluid (PGu 0.02 and PEu 0.01/ml. of the reaction mixture) than in the cell-free extract of the mycelium (PGu 0.008/ml. of the reaction mixture and negligible PME). No *trans*-eliminase activity was detected at pH values from 4.5 to 8.0 with pectin or polygalacturonic acid as substrates.

Course of enzyme secretion. The secretion of polygalacturonase was followed as a function of time. When the fungus had formed a surface deck, samples were harvested periodically and the activity estimated in the culture fluid. The results are presented in Fig. 1. PME was demonstrated qualitatively to be present from the tenth day onwards. Since maximum PG activity was observed between 18 and 20 days, further work was done with 20 day cultures.

Purification of polygalacturonase. Because PME would have interfered with the study of the properties of polygalacturonase, purification of the culture fluid was directed towards removal of this activity. The culture fluid was dialysed against distilled water for 12 h. at 0°, and then treated with 3 vol. of cold acetone. The precipitate formed was collected by centrifugation at 10,000g for 15 min. in a Lourdes refrigerated centrifuge, dissolved in water and centrifuged again to remove insoluble material.

Subsequently, $(\text{NH}_4)_2\text{SO}_4$ fractionation was done. The supernatant obtained after

70% saturation (which contained most of the enzyme activity) was dialysed and used for further purification.

Alumina C γ gel (6 mg./10 ml.) was added (20% by vol.) to the enzyme solution obtained from the previous step. The mixture was stirred for 15 min. in the cold and

Fig. 1. Secretion of polygalacturonase as a function of time. The substrate was 0.5 % polygalacturonic acid in 0.1 M-Na acetate buffer, pH 5.4; 2.5 ml. culture fluid was used/10 ml. reaction mixture (room temperature). ●, Dry wt mycelium; ○, enzyme activity.

Table 1. *Purification of polygalacturonase*

Purification step	Specific activity (PGu/mg. protein)	Recovery (%)	Specific activity (PEu/mg. protein)
Crude culture fluid	0.2	100	0.19
Acetone precipitation	1.3	128.7	0.40
Ammonium sulphate (70 %) (supernatant)	4.6	101.8	Trace
Alumina C γ gel adsorption and elution	12.9	49.2	Nil

centrifuged. The supernatant liquid was inactive and was discarded. The gel was dispersed in a small volume of 0.1 M-potassium phosphate buffer, pH 7.0, and centrifuged after 15 min. Only 49% of the activity was recovered in the supernatant, but specific activity was substantially increased (Table 1).

Use of calcium phosphate gel and isoelectric precipitation did not result in an increase in specific activity.

Substrate specificity. The rate of hydrolysis of pectin and polygalacturonic acid was measured. The polygalacturonic acid was hydrolysed fairly rapidly but pectin was almost resistant to attack (Fig. 2). Prolonged digestion (72 h.) of polygalacturonic acid resulted in 68.7% hydrolysis.

The hydrolysis of the substrate was followed chromatographically. At an early stage polygalacturonic acid was broken down into higher uronides (R_F 0.039, 0.08

and 0.15), which later disappeared with the appearance of mono- and digalacturonides (R_f 0.43 and 0.25 respectively).

Effect of pH on the rate of hydrolysis. The optimum pH for activity of the partially purified enzyme was 5.5 in sodium acetate-acetic acid buffer (Fig. 3).

Effect of temperature on enzyme activity. The enzyme had two temperature optima: one at 50° and the other at 70°. The enzyme was inactivated at temperatures above 70° (Fig. 4). It was thought that the peak at 70° might be due to the presence of an

Fig. 2. Action of endopolygalacturonase on pectin (●) and polygalacturonic acid (○). The substrate concentration was 0.5 % in 0.1 M-Na acetate buffer, pH 5.4; enzyme concentration was 5.76 units for 80 ml. reaction mixture; at 30°. The reaction mixture was covered with a layer of toluene to avoid contamination.

Fig. 3

Fig. 4

Fig. 3. Effect of pH on endopolygalacturonase activity. The reaction mixture (30°) contained 1.0 % polygalacturonic acid, 10 ml.; 1.0 M buffer of specified pH, 2.0 ml.; enzyme, 1.44 units; water to 20 ml.

Fig. 4. Effect of temperature on endopolygalacturonase activity. The reaction mixture contained polygalacturonic acid, 1.0 %, 10 ml.; acetate buffer, 1.0 M, pH 5.4, 2.0 ml.; enzyme, 1.44 units; and water to make up the volume to 20 ml.

isozyme. However, attempts to separate these two activities on a DEAE-cellulose column were not successful.

Effect of various metal ions and inhibitors on enzyme activity. Mill (1966) reported the activation of exopolygalacturonase of *Aspergillus niger* by Hg^{2+} ions. Hence the effect of various metal ions at a final concentration of 1×10^{-3} M on PG activity was tested. The purified enzyme did not require added metal ions for its activity. Except for Fe^{2+} and Fe^{3+} all metal ions tested inhibited PG activity: Hg^{2+} , Co^{2+} and Cu^{2+} by 60% and Ca^{2+} , Mg^{2+} , Mn^{2+} and Zn^{2+} by 24%, 18%, 13% and 12.5% respectively. Neither -SH group binding agents like iodoacetate (1×10^{-3} M), *p*-chloromercuribenzoate (1×10^{-5} M) and *N*-ethylmaleimide (1×10^{-4} M) nor sodium fluoride (1×10^{-3} M) and sodium azide (1×10^{-4} M) had any effect.

Effect of fungicides on enzyme action. These fungicides were tested at a final concentration of 0.1%. Blitox-50 (50% Cu as oxychloride) inhibited the enzyme by about 85% whereas maneb and zineb inhibited activity by about 7% and 21% respectively.

DISCUSSION

The secretion of endopolygalacturonase (EC 3.2.1.15 polygalacturonide glycanohydrolase), i.e. the enzyme which attacks polygalacturonic acid, was demonstrated. The chromatographic study of the products of the action of the enzyme on polygalacturonic acid showed that this enzyme cleaved the chain at random. The enzyme had only feeble action on pectin, which showed that it was a polygalacturonase rather than a polymethylgalacturonase.

The method of purification described resulted in an approximately 65-fold purification of the endo-PG from the culture fluid of *Ganoderma lucidum*.

The *Ganoderma* PG had a very low specific activity (12.9 PGu/mg. protein) when compared with other PGs of yeast and fungal origin, e.g. 179 PGu/mg. protein for the endo-PG of *Saccharomyces fragilis* (Phaff & Demain, 1956) and 1261, 502 and 409 PGu/mg. protein (converted from 7882, 3140 and 2560 PGu/mg. of total nitrogen respectively) for three endo-PGs from *Coniothyrium diploidella* (Endo, 1964). However, the specific activity of *Ganoderma* PG closely resembled that of tomato and avocado PGs (Patel & Phaff, 1960; Reymond & Phaff, 1965).

Like the endo-PGs of *Saccharomyces fragilis* (Phaff & Demain, 1956), *Aspergillus saitoi* (Yamasaki, Yasu & Arima, 1966) and *Coniothyrium diploidella* (Endo, 1964) *Ganoderma* PG hydrolysed polygalacturonic acid to an extent of about 69%; the reaction was fast and linear until 25% of the polygalacturonic acid was hydrolysed.

The optimum pH (5.0 to 5.5) for the *Ganoderma* PG was slightly higher than the values reported for other fungal and yeast PGs, which range from 3.3 to 4.8 (e.g. Demain & Phaff, 1954; Luh & Phaff, 1954; Mill & Tuttobello, 1961; Endo, 1964).

With optima at 50° and 70°, the *Ganoderma* PG had remarkable thermal stability: polygalacturonases from most sources are rapidly inactivated at temperatures of 60° and above, although a fraction of tomato enzyme has been found to resist 100° for 5 min. (McColloch & Kertesz, 1948).

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