J. Indian Inst. Sci. 63 (C), Dec. 1981, Pp. 157-166

Partial purification and some properties of a polygalacturonase produced extracellularly by *Alternaria alternata* (Fr.) Keissl

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Abstract

A polygalacturonase was purified to about 480 fold from the culture filtrate of *Alternaria alternata* (Fr.) Keissl. The partially purified enzyme had a pH optimum of 5·2 and activation energy of 4·77 K cal/mole for polygalacturonic acid (sodium salt). V_{max} and K_m were found to be 333 μ moles of galacturoric acid liberated per hour at 37° and 0·106% (2·9 × 10^{-5} M) polygalacturonic acid respectively. The enzyme was only slightly activated by Ca^{+2} and Co^{+2}. Tannic acid inhibited the activity but chlorogenic, caffeic and ferulic acids did not have any effect. The purified enzyme did not utilize low chain length oligogalacturonides indicating that it might be an endopolygalacturonase.

Key words: Pectolytic enzyme, pathogenesis in fruits, host-pathogen relationship.

1. Introduction

Pectolytic enzymes are known to be produced by a number of organisms and have received considerable attention also because of their technological applications[1]. Fungal enzymes have received particular attention in this respect. Apart from their industrial importance, pectolytic enzymes have also been implicated in pathogenesis in fruits by various organisms and have been extensively studied from this angle also. Some knowledge of the behaviour of these enzymes (*e.g.*, optimum conditions for maximum activity) is likely to lead to a better understanding of mechanisms involved in host-pathogen relationship. It might also help in a more efficient use of these enzymes in industry.

Among fungi, a number of *Alternaria* species have been shown to produce polygalacturonase (PG) activity, both *in vitro* and in infected plant tissues[2,3]. A strain of

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Alternaria alternata (Fr.) Keissl, which was isolated by the authors[4] in this laboratory from infected Mandarin oranges was found to produce considerable PG activity under suitable culture conditions[5]. The present paper describes partial purification of such a polygalacturonase and some of its kinetic properties.

2. Material and methods

2.1. *Organism*

A. alternata was maintained on potato dextrose agar. The culture was grown in a medium containing 0·5% pectin and 0·5% cellulose powder[5]. The initial pH of the medium was adjusted to 3·0. The medium was seeded with 4% inoculum and incubated at 25° for 96 hours.

2.2. *Enzyme assay*

PG activity was determined by measuring the liberation of reducing groups. One ml of reaction mixture contained suitably diluted enzyme, 50 mM acetate buffer pH 5·2 and 0·25% polygalacturonic acid sodium salt (Sigma Chemical Co., U.S.A.). Boiled enzyme was used in control tubes. The incubation temperature was 37° and time 1 hr. Galacturonic acid release was measured by arsenomolybdate method[6], taking galacturonic acid monohydrate (Sigma) as the standard.

2.3. *Determination of proteins*

Proteins in the enzyme preparations were estimated by the spectrophotometric method (E 280/E 260) of Warburg and Christain[7].

2.4. *Purification of PG*

All the operations were carried out at 0°–4° unless otherwise stated.

Step 1 : The fluid from the culture bottles was pooled, filtered through Whatman No. 41 and centrifuged to get a clear solution. This was designated as crude enzyme.

Step 2 : 250 ml of the crude enzyme was dialysed against distilled water for 20 hours and were concentrated against sucrose to a final volume of 39 ml. This preparation was designated as dialysed and concentrated enzyme.

Step 3 : 37 ml of the enzyme obtained in step 2 was dialysed for a brief period against 0·02 M acetate buffer pH 5·0 and was loaded on a DEAE cellulose column

of 1·4 × 22 cm, previously equilibrated with 0·02 M acetate buffer, pH 5·0. The proteins were eluted with a stepwise gradient as follows;

0·02 M acetate buffer pH 5·0 containing no NaCl—30 ml

0·02 M acetate buffer pH 5·0 containing 0·03 M NaCl—30 ml

0·02 M acetate buffer pH 5·0 containing 0·06 M NaCl—60 ml

0·02 M acetate buffer pH 5·0 containing 0·08 M NaCl—80 ml

0·02 M acetate buffer pH 5·0 containing 0·18 M NaCl—140 ml

10 ml fraction was collected. Fractions having maximum PG activity (Fig. 1) were pooled, concentrated against sucrose and dialysed for a brief period against 0·02 M acetate buffer pH 5·0. The final volume was 16·4 ml. This constituted DEAE cellulose fraction.

Step 4 : 16 ml of the enzyme obtained in step 3 was loaded on a Sephadex G-75 (Pharmacia, Uppsala, Sweden) column 1·4 × 33 cm, previously equilibrated with 0·02 M acetate buffer pH 5·0. The elution was done by the same buffer without altering ionic strength. 3 ml fractions were collected. Fractions showing maximum activity (Fig. 2) were pooled and concentrated. This constituted the purified enzyme.

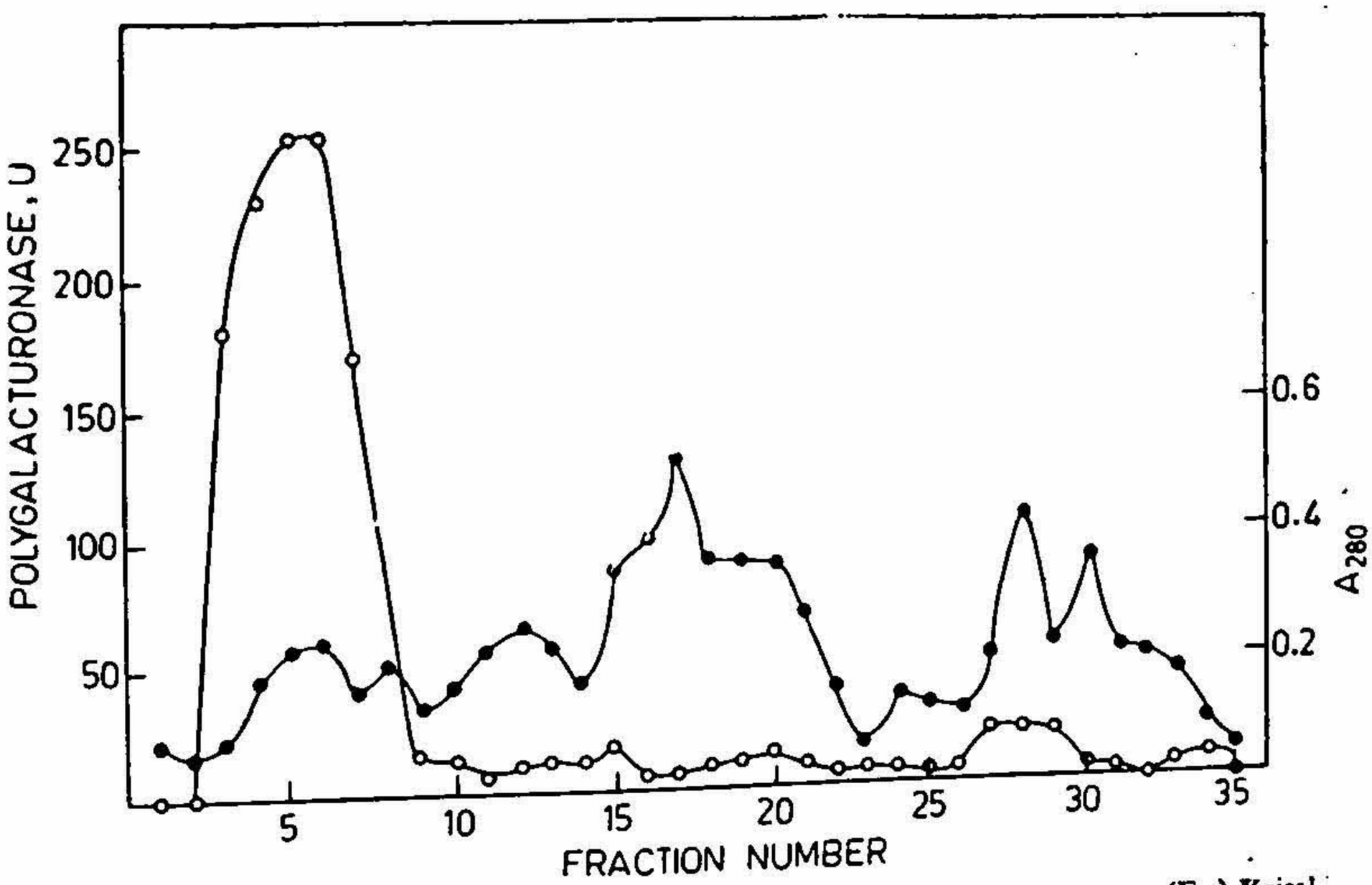

FIG. 1. DEAE cellulose chromatography of polygalacturonase from *A. alternata* (Fr.) Keissl.
○-○ PG activity (U/ml) ●-● A280.

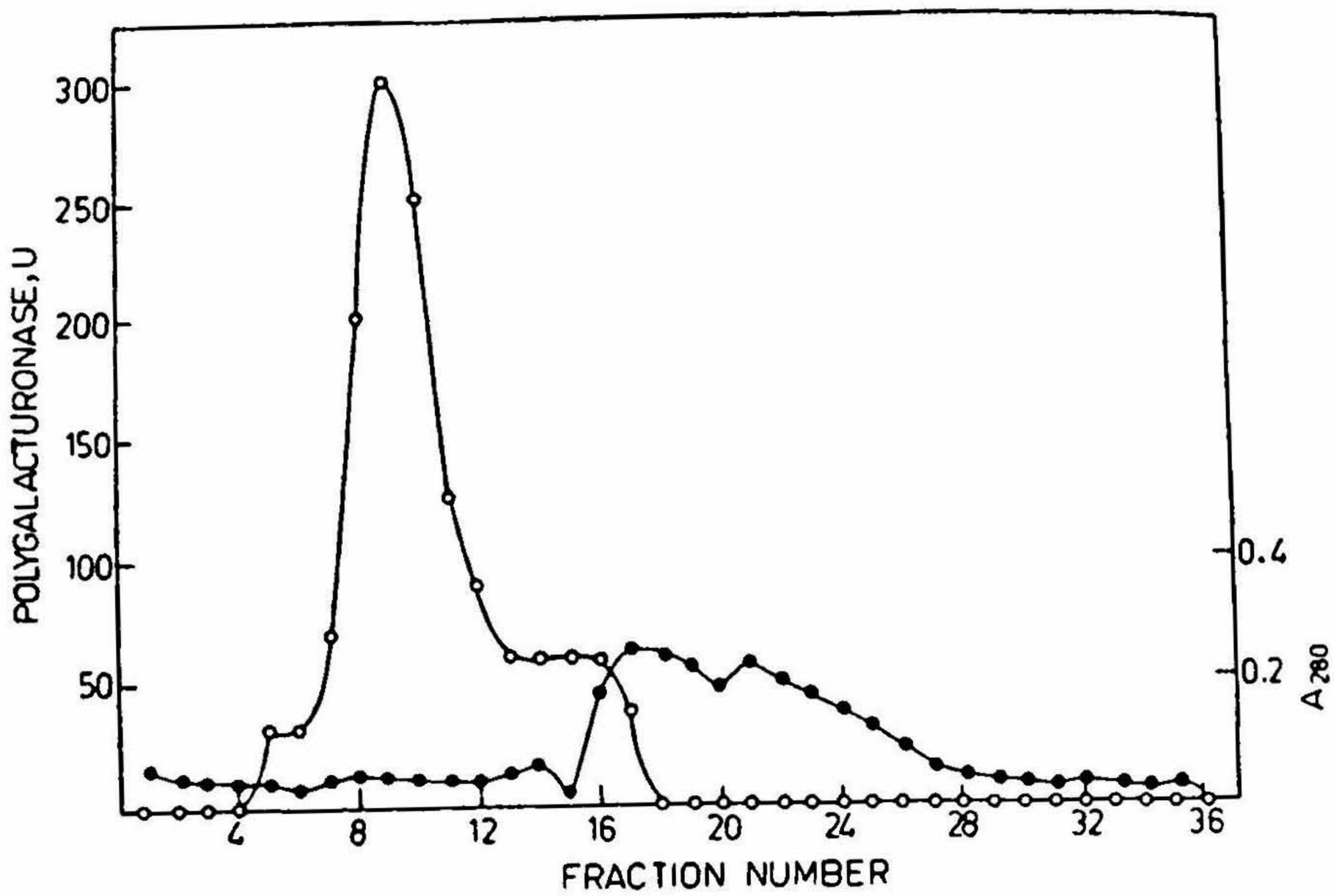

FIG. 2. Sephadex G-75 chromatography of polygalacturonase from *A. alternata* (Fr.) Keissl. O–O PG activity (U/ml) ●–● A280.

2.5. *Polyacrylamide gel electrophoresis*

The gel electrophoresis was performed on 0·6 × 7 cm gel of 7% polyacrylamide using the alkaline buffer system of Davis[8]. The gels were stained in 1% Amido black in 7% acetic acid[9].

3. Results

3.1. *Enzyme purification*

The studies indicated that it is possible to extensively purify the PG from *A. alternata* (Fr.) Keissl by the procedure described. All the steps given in Table I were repeated several times and the results were found to be reproducible. The final preparation was apparently electrophoretically homogeneous.

3.2. *Stability of the enzyme*

The PG was stable for 15 days if stored at −20°. Repeated freezing and thawing decreased the activity to a considerable extent.

Table I

Summary of purification of polygalacturonase from culture filtrates of *A. alternata* (Fr.) Keissl

Step	Volume (ml)	Proteins (mg/ml)	Total proteins	Units (U/ml)	Total units	Specific activity (U/mg proetin)	Purification	Yield %
Crude enzyme	250	0·780	195·0	28·6	7065	37·0	...	100·0
Dialysis and concentration	39	0·714	28·0	158·51	6182	222·0	6·0	87·5
DEAE-cellulose chromatography	16·4	0·333	5·5	363·0	5952	1082·0	29·2	84·2
Sephadex G-75 chromatography	9·0	0·014	0·126	243·0	2187	17857·0	481·2	30·96

3.3. *Thermal stability*

The PG was stable for 4 minutes when the enzyme was heated at 55°, but thereafter its activity decreased very rapidly.

The effect of pH on thermal stability was also studied. The enzyme was adjusted to various pH values by diluting it with 0·05 M sodium acetate solutions, to have a pH range of 3–7, before heating. These were then placed at various temperatures between 37°–60° for 5 minutes ; and residual PG activity was measured. The PG was most stable to heat at pHs between 4–5 and the least stable at pH 7 (Fig. 3).

3.4. *Effect of pH*

The hydrolysis of polygalacturonic acid by PG was found to be optimum at pH 5·2 (Fig. 4). In this respect the purified enzyme appeared to be different from the crude enzyme, which had a pH optimum at 4·4. Moreover, the crude enzyme under the standard assay conditions showed linear relationship with time up to 24 hours, but the purified enzyme showed linearity only up to 1·5 hours. The optimum temperature was, however, the same for both the enzymes,

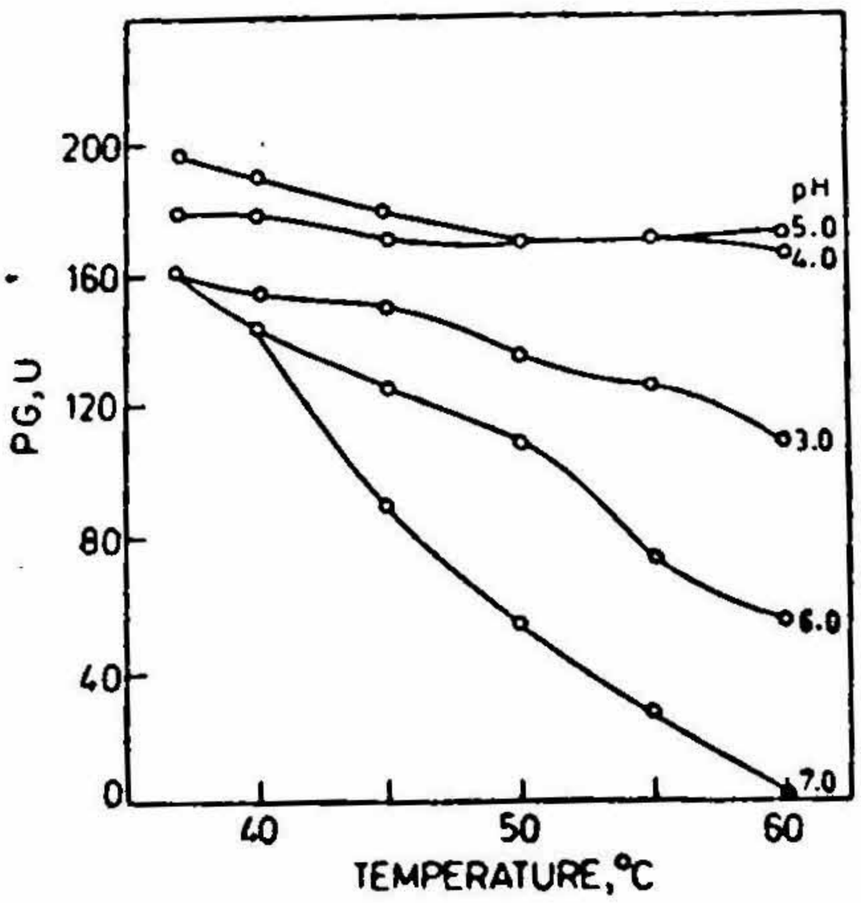

FIG. 3. Effect of pH on thermal stability of polygalacturonase from *A. alternata* (Fr.) Keissl. Activity expressed as U/ml.

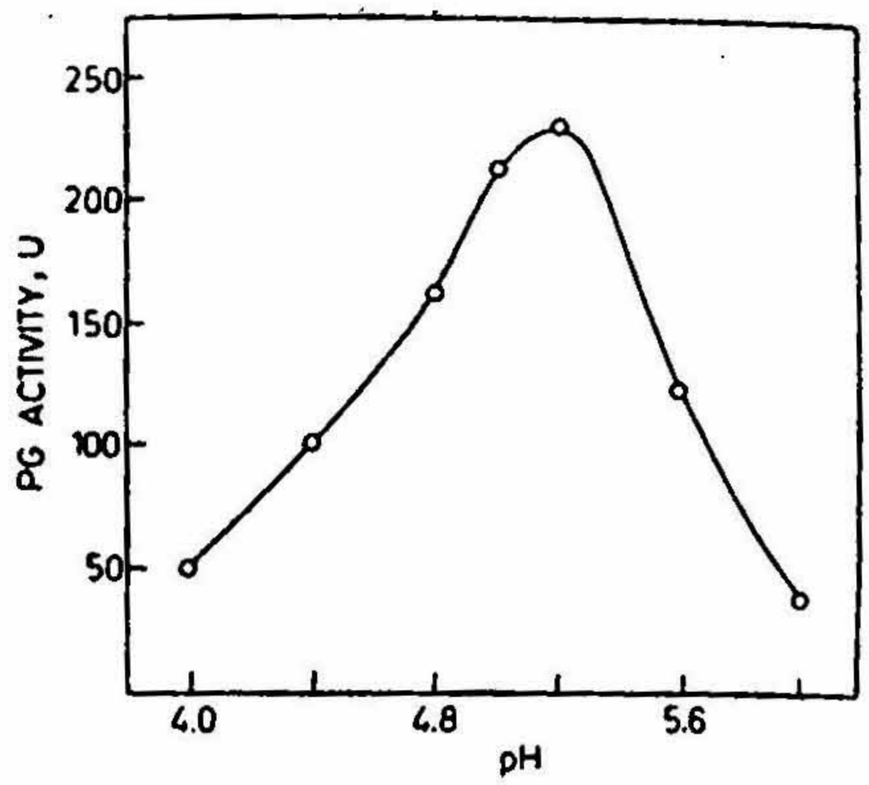

FIG. 4. Effect of pH on activity of polygalacturonase (U/ml) from *A. alternata* (Fr.) Keissl.

3.5. *Effect of temperature*

The PG had an optimum temperature of 50°. The activation energy (Ea), calculated by plotting Arrhenius plot, was found to be 4·77 Kcal per mole (Fig. 5).

3.6. *Effect of substrate concentration*

Kinetic constants for PG were determined by plotting Lineweaver and Burk plot. Polygalacturonic acid was used as substrate (Fig. 6). V_{max} was calculated to be 333 μ moles galacturonic acid per hr at 37°. Assuming the molecular weight of polygalacturonic acid to be 35400[10], K_m for PG from *A. alternata* was $2{\cdot}9 \times 10^{-5}$ M.

3.7. *Effect of ions*

Effect of different mono and divalent ions at 1 mM concentration on PG activity was studied (Table II). All the ions, except Ca^{+2} and Co^{+2}, inhibited the activity to varying extents. Hg^{+2} and Cu^{+2}, were completely inhibitory. Ca^{+2} and Co^{+2} had almost no effect on PG activity at 1 mM concentration ; however, both these ions stimulated activity at 0·5 mM concentration. The stimulation in activity was about 6% for Ca^{+2} and 15·6% for Co^{+2}. Both the ions were inhibitory at 5 mM concentration.

3.8. *Effect of phenolic inhibitors*

Response of PG activity to tannic, chlorogenic, caffeic and ferulic acids at 1 mM concentration is shown in Table III. Only tannic acid inhibited the activity. At 1 mM level, the inhibition was about 94% and at 0·1 mM level about 44%.

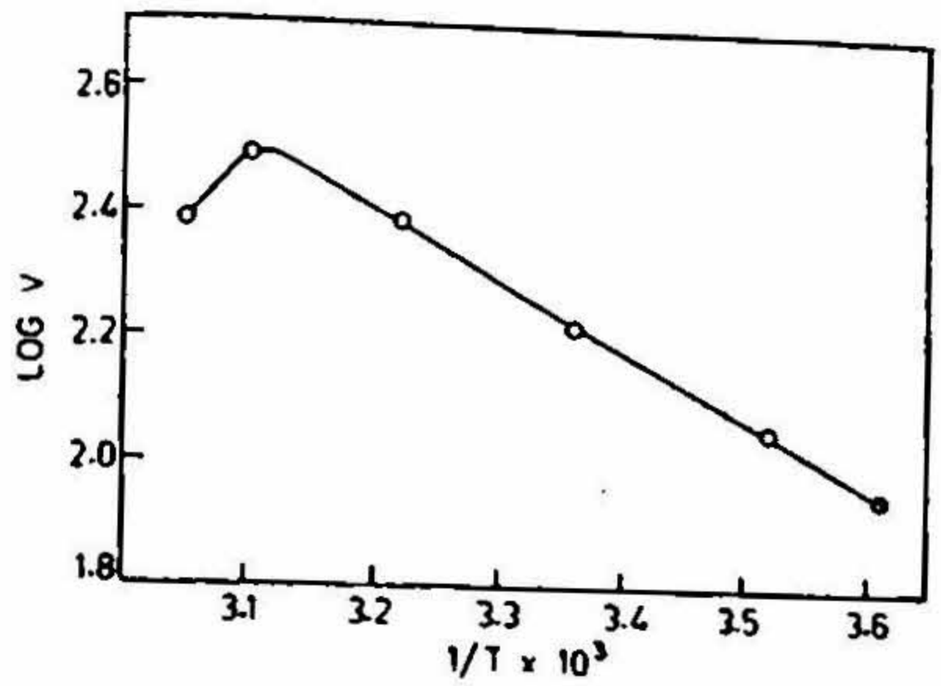

FIG. 5. Effect of temperature on activity of polygalacturonase from *A. alternata* (Fr.) Keissl.

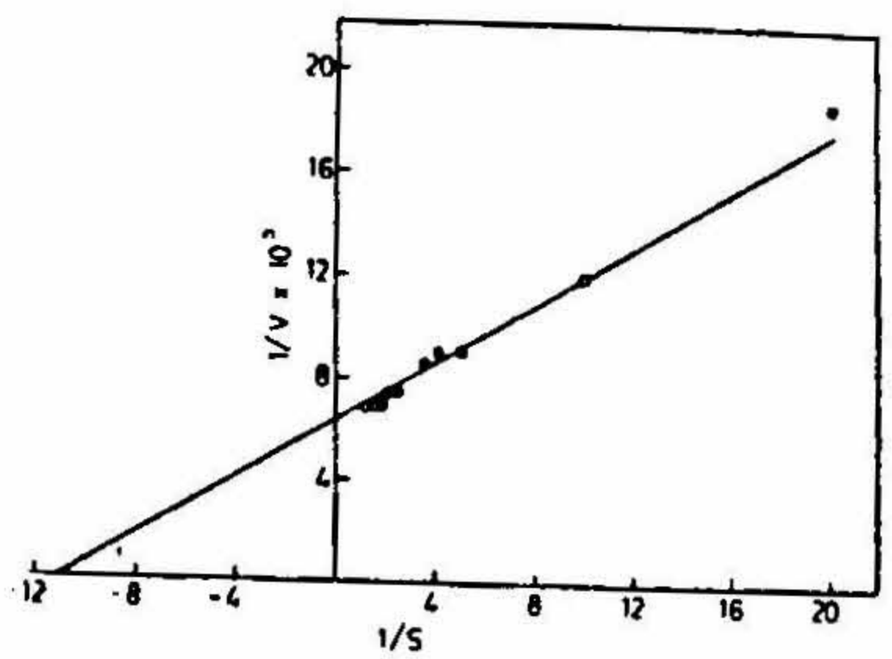

FIG. 6. Lineweaver–Burk plot for polygalacturonase from *A. alternata* (Fr.) Keissl.

3.9. *Effect of oligogalacturonides*

Utilization of various oligogalacturonides by the PG were studied by replacing polygalacturonic acid in the standard reaction mixture by di-, tri-, tetra- and pentagalacturonic acid (Table IV). The enzyme did not break down di-, tri- and tetragalacturonic acids to any significant extent. Pentagalacturonic acid was however utilised, but to a much lesser extent than polygalacturonic acid.

4. Discussion

Results presented in the present paper indicate that it is possible to purify polygalacturonase produced extracellularly by *A. alternata*, a soft rot organism of Mandarin oranges.

Although many reports on purification of PG from different fungal sources have appeared in literature[11–13], not much work appears to have been done on *Alternaria*. Moreover, the purification reports tend to be scanty in kinetic data. Since the present strain causes soft rot in oranges and many other fruits, and since pectolytic enzymes are important in onset and progress of the rot, studies on the nature of pectolytic enzymes elaborated by *A. alternata* were thought pertinent. The present study was thought to be desirable to undertake from another view point also. Pectinases are immensely important in food industries and exploration of a new source of the enzyme is always welcome.

On account of scanty work reported on PG of *Alternaria* species, discussion of the properties of the enzyme of *A. alternata vis-a-vis* other reports is difficult. However, when considered in the light of the work on other fungi it is apparent that the degree of purification achieved in the present investigations is comparable to those reported[12,13]. Purified PG when subjected to gel electrophoresis appeared homogeneous, although

Table II

Effect of different ions on polygalacturonase from *Alternaria alternata* (Fr.) Keissl

Ion	Conc. (mM)	% activity
None	...	100
Hg^{2+}	1	0
Cu^{2+}	1	3
Mn^{2+}	1	10
Mg^{2+}	1	15
Fe^{2+}	1	25
Zn^{2+}	1	43
Cd^{2+}	1	45
Ca^{2+}	0·005	100
,,	0·05	102
,,	0·10	104
,,	0·5	106
,,	1·0	96
,,	5·0	31
Co^{2+}	0·005	100
,,	0·05	100
,,	0·1	100
,,	0·5	116
,,	1·0	100
,,	5·0	22

Table III

Effect of some phenolic compounds on polygalacturonase from *Alternaria alternata* (Fr.) Keissl

Addition (1 mM)	% activity
None	100
Tannic acid	6
Tannic acid (0·1 mM)	46
Chlorogenic acid	9
Ferulic acid	96
Caffeic acid	100

Table IV

Effect of oligogalacturonides on polygalacturonase from *Alternaria alternata* (Fr.) Keissl

Substrate	% activity
Polygalacturonic acid	100
Pentagalacturonic acid	15
Tetragalacturonic acid	N.S.
Trigalacturonic acid	N.S.
Digalacturonic acid	N.S.

N.S. : Not significant.

other tests of homogeneity such as isoelectric focussing and rechromatography are needed to confirm it.

A notable difference observed is that in most of the earlier reports, the protein content of the culture filtrate was quite high as compared to our enzyme. This might be an advantage since one starts with lesser amount of extraneous proteins. The purified pH showed a shift in optimum pH from 4·4 to 5·2. It has been suggested that several factors may be responsible for this. Firstly, it is possible that during the purification process some substance(s) is eliminated, which otherwise has some bearing on pH optimum. Secondly, ratio of endo-PG to exo-PG changes during purification. It has been observed by Mussel and Strouse[14] in their studies on PG from *Verticillium albo-atrum*, that culture filtrates containing different ratios of exo-PG to endo-PG do in fact

exhibit different pH optima when assayed as a mixture. Swinburne and Corden[15] have suggested that properties of *in vitro* PG preparations may change after purification.

Different values for activation energy (Ea) have been reported by different workers. Even different strains of the same species have been found to differ in the Ea. Ea of 8·3 K cal/mole has been reported for a strain of *A. niger*[16] whereas Nyeste *et al*[17] have calculated it to be 16·065 K cal/mole for a PG from strain 21 of *A. niger*. A PG from *Rhizopus arrhizus* has an Ea of 11·9 K cal/mole. Compared to these the PG of *A. alternata* has a low Ea *i.e.* 4·77 K cal/mole.

A wide range of other kinetic constants has been reported for PG preparations from various sources. *Rhizopus arrhizus* endo-PG has a V_{max} and K_m of 1·43 μ moles of galacturonic acid/min and 0·054% polygalacturonic acid respectively[13]. The two endo-PGs from *Trichoderma koningii* have K_m values of 0·81 and 0·86 g per litre[18]. V_{max} for a PG of *Verticillium alboatrum* has been reported to be 2150 μ moles galacturonic acid per min/mg protein[19]. The K_m was 0·15% polygalacturonic acid. Microbial PG seems to have generally high K_m values than the PG from fruits and grains. PG from oat[20], pear[21] and cucumber[22] have been reported to have K_m values of 6 μM, 2 μM and 34 μM respectively.

The PG from *A. alternata* appears to be affected by phenolic compounds in a way which is similar to that reported by Negoro[23] and Liu and Luh[13].

Our observation that the purified PG utilizes polygalacturonic acid readily, pentagalacturonic acid to a much less extent and fails to break down to tetra-, tri-, and di-galacturonic acid indicates that the enzyme might be an endo-PG as suggested by Liu and Luh[13]. Studies on breakdown products of the enzyme reaction might throw further light on the properties of the enzymes.

Acknowledgement

The authors wish to thank Prof. C. H. Chakraborti, Head of the Department, for his interest in the work and the University Grants Commission, New Delhi, India, for financial assistance. They are grateful to Dr. R. Pressey, Russel Agricultural Research Centre, Athens, Georgia, U.S.A., for a generous gift of oligogalacturonides.

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