INTERNATIONAL JOURNAL OF ADVANCED BIOLOGICAL RESEARCH

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ISOLATION AND PURIFICATION OF ENDO POLYGALACTURONASE PRODUCED BY ALTERNARIA CEPULAE

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ABSTRACT
The extracellular endopolygalacturonase from *Alternaria cepulae* causing leafblight disease in Onion was isolated and purified. The crude enzyme extract obtained in onion medium was precipitated with 50-95% ammonium sulphate at pH 5.0. The isoenzymes were separated on DEAE - Cellulose column with 0.01 M Phosphate buffer at pH 7.0. Further purification was achieved in ultrogel ACA 44 (LKB) column. The endo PG I and endo PG II have migrated as a single band on PAGE & SDS - PAGE. These isoenzymes have an isoelectric point of 6.7 and 7.1 and give single immunopreciptin line.

KEY WORDS: endopolygalacturonase, isoenzymes, DEAE, PAGE & SDS - PAGE etc.

INTRODUCTION

Pectic enzymes play an integral part in pathogenesis of a wide spectrum of plant diseasesAmong these enzymes, the occurrence and production of polygalacturonase (poly x 1-4 D galacturonideglycano hydolase EC3.2.1.15)by many fungi during disease development has been widely ascertained (Bateman and Millar, 1966 Shiro,etal,1984, Bashan, etal,1985). *Alternaria cepulae* is known to produce endopolygalacturonase (Ponnappa *et al* 1977). A study was therefore planned to purify the enzyme.

MATERIALS AND METHODS

Fungi

The fungus *Alternaria cepulae* (Ponnappa) employed in the present study was isolated from the infected onions. The isolate was maintained on PDA at 32± 2°C. This was subcultured at intervals of 3 months and the stock cultures were maintained at 4°C.

Enzyme preparation

One kg samba onions were cut into small pieces. This was taken along with500ml of water in 5lit haffkins flask. The flask was sterilized with the contents at 15 lb pressure in Barnslead autoclave (Boston) for 20 minutes. Then the flask was cooled to room temperature (32±1°C) and a slant of A. cepulae was inoculated into the onion medium .After 16 days the contents along with 0.25 M NaCl were blended in a waring blender for 5 seconds at 4°C. The blended juice was filtered and centrifuged at 20.000 rpm for 20 minutes at 4°C. The clear supernatant was dialysed against 2 lit of 0.01 M phosphate buffer atpH 7.0 for 48 hours with 2 changes at 4°C. Ammonium sulphate fractionation. To 1 lit of dialysed supernatant solution, ammonium sulphate (AR) was added slowly with a constant stirring in a magnetic stirrer for 4 to 6 hours at 4° C to give 0-40%, 40-80%, 80-95% saturation. After the desired percentage of saturation the contents of each step was centrifuged at 15,000 rpm for 20 minutes at 4°C. The precipitate obtained was dissolved and dialysed against 0.01 M phosphate buffers at pH 7.0 containing 0.1 M NaCl with 3 changes of buffer.

Ion exchange column chromatography

50 ml of the dialysed samples from 80-95% $(NH_4)_2 SO_4$ precipitate were applied to a DEAE cellulose column(3 x 25 cm) equilibrated with 0.01 M phosphate buffer (pH 7.0). They were eluted with the same buffer containing stepwise gradient of 0.05 M NaC1 at the flow rate of 9ml h-1. 3 ml fractions were collected. The individual fractions were read at 280 nm and EPG activity was estimated.

EPG Assay

Endo polygalacturonase activity was estimated by the method of Nelson somogyi (1944 and 1952).

Estimation of protein

Protein content was determined by the method of Lowry *et al* (1951) using crystalline bovine serum albumin (BSA) as standard.

Tests for homogeneity

PAGE was carried out according to the methods of DAVIS(1964). SDS-PAGE was done by adopting the procedure of Laemmli (1970). Doubleimmunodiffusion was carried out by the method of Ouchterlony (1973).
Immuno diffusion electrophoresis was performed according to the method of Graber and Burtin (1964) Isoelectricfocussing was carried out in a semipreparativemanner based on the method of Wrigley (1971)

RESULTS

Fig 1: shows theelution profileofDEAE - Cellulose cloumn chromatography of EPG. The precipitate obtained

between 80-95% (NH4)2 SO4 fraction was dialysed and applied on DEAE - Cellulose column. The stepwise gradient elution shows that the first fraction of EPG activity (designated as EPG x) eluted at the concentration of 0.1 M NaC1 and the second fraction of EPG activity (Designated as EPG II) eluted at the concentration of 0.2 M NaCl.

Elution profile of EPG I from DEAE - Cellulose column after pooling and dialysis against 0.01 M phosphate buffer (pH 7.0) was lyophilized. A small amount was dissolved in the same buffer and loaded on ultrogel column (Fig2) shows two protein fractions but EPG activity is seen only in 1st fraction, ie from 36th to 52nd fraction.

The second fraction of DEAE column after conducting the same procedure as described above was loaded to the ultrogel column. The elution profile (Fig 3) shows only the one peak which contains both protein and EPG activity. The EPG II activity is seen from 48th to 56th fraction.

A summary of purification presented in Table 4 reveals that EPGI is purifiedto 245 fold with 26% yield. EPG II is purified to 270 fold and with 22% yield.

From each step of purification the enzyme samples weretested for homogeneityusing PAGE and SDS-PAGE. After final purification EPGI shows only one band in PAGE and SDS - PAGE (Fig 6). Similarly EPG II also shows single band in PAGE (Fig 7) and SDS - PAGE (Fig 8).

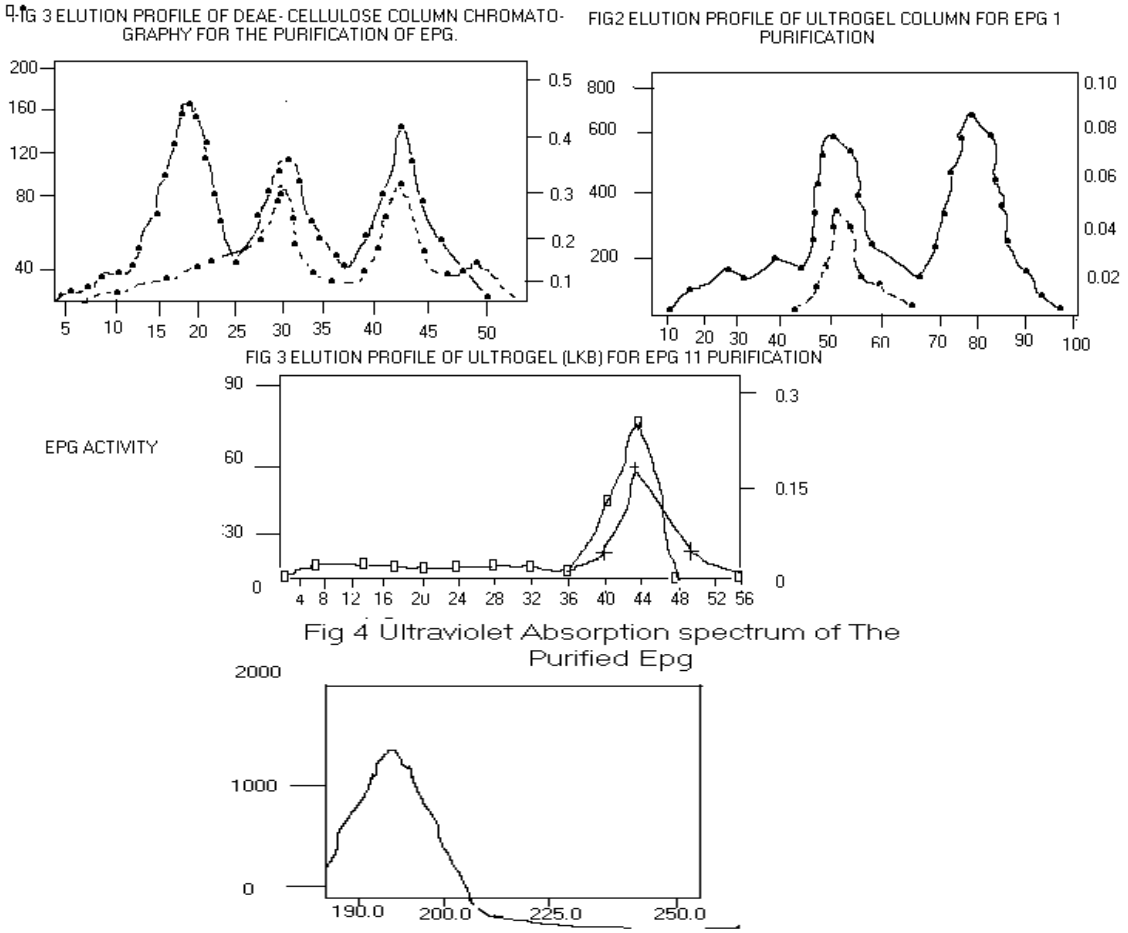


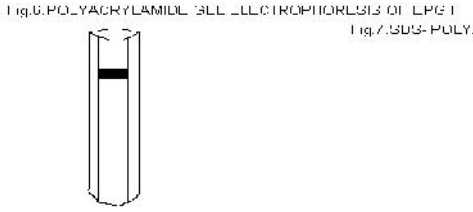
TABLE 1: Ammonium Sulphate precipitation of crude enzyme preparation of EPG from natural onion medium

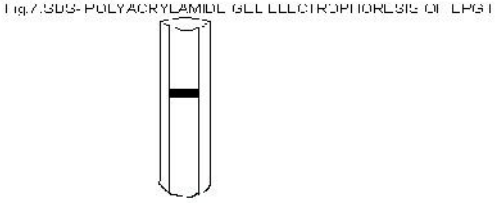
Sl No	Percentage of $(NH_4)_2$ SO_4 (%)	Volume(ml)	EPG activity units up of gal. acid rel/ml/30 min	Protein content µg/mi	Spcific activity in units/ug
1	Crude enzyme	900	56.4	71.2	0.79
2	0-40	30	1.32	26.4	0.05
3	40-80	40	2.04	29.1	0.07
4	80-95	50	976.4	76.9	12.0

TABLE 2: Purification of endopolygalacturonase

Steps	Volume (ml)	Total Protein (µg)	Total endoPG Activity units (µg of gal.acid rel)	Specific activity Units/µg protein	Purifocation fold	Yield (%)
Culture filtrate(supernatant)	900	64080	50760	0.79	1	100
80-95% $(NH_4)_2 SO_4$ Precipitation DEAE-column	50	3845	48820	12.7	16.0	96.18
EPG I-fraction	138	201	21618	107.55	135.5	42.58
EPG II - fraction	40	162	20825	128.54	161.34	41.01
Ultrogel column EPG 1-fraction	34	68	13285	195.36	245.18	26.16
EPG II –fraction	16	52	11226	215.88	270.93	22.11

Determination of isoeletric point in a semipreparative manner of EPGI is shown in Fig. 9. It indicates a single peak of EPG activity between 11th and 12th fractions at isoelectric pH of 6.7. The semipreparative isoelectric focussing of EPG II is shown in Fig10. It shows a single of EPG activity between fractions 16 and 17 and its isoelectric pH is 7.1.The ouchterlony's double immune diffusion pattern indicatesthe homogeneity of the EPG I and EPG II (Fig11). The highly purified enzyme gave a single immunoprecipitin line in reflected light when reacted with its rabbit antiserum showing the presence of only one component.Graber & Burtin qualitative analysis by immunoelectrophoresis also confirmed the homogeneity of the enzyme as depicted in Figure 12.





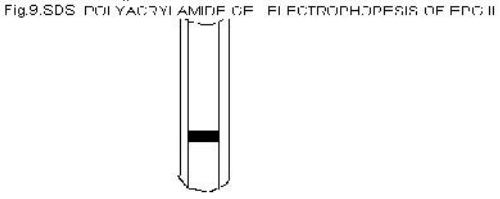


Fig 10 DETERMINATION OF ISOELECTRIC POINT OF EPG I

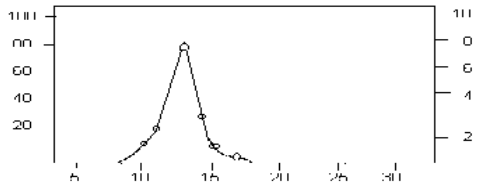


Fig 11 DETERMINATION OF ISOELECTRIC POINT OF EPG II

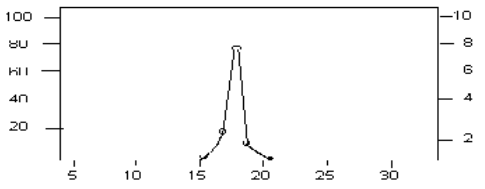


Fig 10 DETERMINATION OF ISOELECTRIC POINT OF EPG I

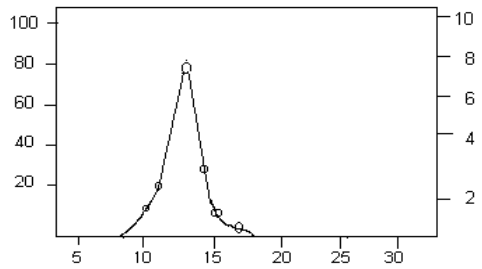


Fig 11 DETERMINATION OF ISOELECTRIC POINT OF EPG II

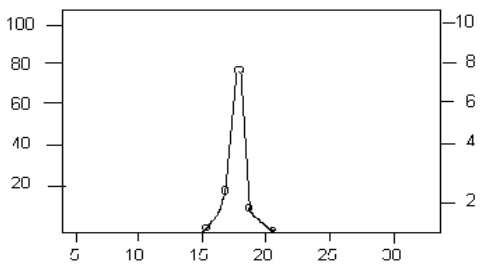


Fig 12. OUCHTERLONY'S DOUBLE DIFFUSION PATTERNS OF PURIFIED EPG I

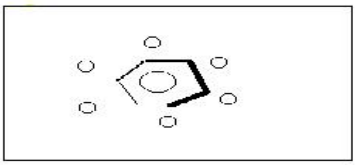


Fig 13 GRABER AND BURTIN IMMUNOELECTROPHORESIS PATTERN OF PURIFIED EPG I

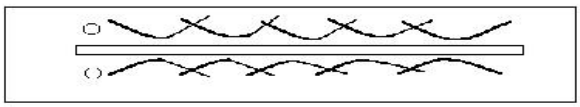


Fig14. OUCHTERLONY'S DOUBLE DIFFUSION PATTERNS OF PURIFIED EPG II

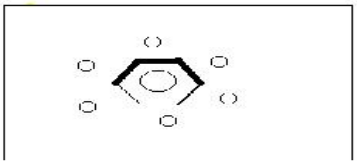
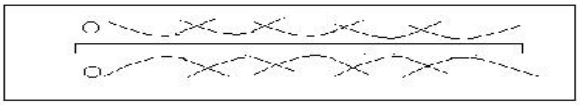


Fig.15. GRABER AND BURTIN IMMUNOELECTROPHORESIS PATTERN OF PURIFIED EPG I



DISCUSSION

Multiple formsof endopolygalacturonase have been reported in leaf spots,blights,wilt disease of plants (Endo,1964, Fielding *et al*., 1969, pressey *et al*., 1973, Hislop *et al*., 1974, Bartheetal, 1981 Scala, 1983 and Arinze, 1985) The DEAE Cellulose column treatment at pH 5.0 selectively removed the brown pigments from the crude enzyme extract giving appreciable increase in specific activity. Similar affinity column was used for thepurification by Pressay, 1973, Strand, *et al*, 1976 and Takahashi, 1985. The DEAE - Cellulose column chromatography at pH 7.0 selectively adsorbed the entire EPG.

The adsorbed enzyme was eluted from the stepwise column gradient as a major fraction (Fig1). Similar elution pattern was reported for the purification of EPG (Urbanek,*et al*, 1975, Magro *et al*., 1980, Hoffman, *et al*, 1982 and York, *et al*, 1986).When crude enzyme from *A. cepulae* was subjected sequentially to six different treatments, the purified EPG I having 245 fold increase in specific acitvity with 26.16% yield & EPG II having 270 fold increase in activity with 22.11% yield were obtained (Table 4)

The results of polyacrylamide gel electrophoresis & SDS-PAGE (Fig 5, 6, 7, and 8) suggest that the purified enzyme ishomogeneous. Isoelectricfocussing pattern (9 & 10) also confirmed the previous findings of the homogeneity of the enzyme. The presence of a single proteinband suggests that EPGIandIIconsists of a single polypeptide chain (Cervons *et al.*, 1977).The immunodiffusion & immunoelectrophoresis results showing single precipition line confirm the homogeneity of the purified EPG

ACKNOWLEDGEMENT

The authors are grateful to Dr. S. C. Dhar and Dr. R. Puvanakrishnan, Scientists at the Department of Biotechnology, CLRI, Chennai for Laboratory facilities and Useful suggestion and UGC, NewDelhi for research grant.

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