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PURIFICATION OF INDOLE ACETIC ACID OXIDASE PRODUCED BY *ALTERNARIA CEPULAE* DURING HYPERAUXINY OF LEAFBLIGHT DISEASE OF ONION

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ABSTRACT
Indole acetic acid oxidase IAAO, an auxin degrading enzyme from *Alternaria cepulae* was estimated in infected leaves of Onion as well as in culture filtrate of the pathogen.The enzyme was isolated and fractionated by employing several techniques was purified to 85.5 fold with 58.6% yield. The homogeneity of the enzyme was confirmed by PAGE, SDS-PAGE, Isoelectricfocussing, Immunodiffusion and Immuno electrophoretic techniques.

KEYWORDS:-*Alternaria cepulae*, CM-Trisacryl column, Ultrogel column.

INTRODUCTION

During Leaf blight disease of Onion, IAA concentration was increased in the blight areas of Onion leaves (Annadurai,1989, 1996, 1998, 1999, 200). It occurred during the first 16 days of infection. Shaw and Hawkins (1958) found 214 fold increase of auxin in wheat infected by *Puccinia graminis* and 5 fold increase of IAA in barley infected by *Erisiphe graminis* (Fric 1975). Pilet (1953) reported that the ratio of IAA equivalents of diseased and healthy tissues per kg fresh weight was 39.7; 7.6. This excess production of auxin is known pathogenesis (Pegg and Selman, 1959, Sequeira and Kelman,1962, Matta and Gentile, 1968). Hyperauxiny is not conducive to the growth of pathogen and hence the pathogen produces auxin degrading enzyme and hence the pathogen produces auxin degrading enzyme for its survival (Annadurai, 1989). Indole acetic acid oxidase is one of the auxin degrading enzyme (Krupasagar,1969,Annadurai,1989). The mould *Omphalia flavida* was reported to produce IAAO while infecting on *Coffea arabica* and *Coleus blumei* (Sequeira and Steeves, 1954).*Verticillium dahliae* on *Capsicum annum* (Srivastava *et al*.,1962), *Fusarium oxysporum* on *Mangifera indica* (Kumar *et al*., 1960), *Protomyces macrosporus* on Coriandrumsativum (Tayal *et al*., 1981). *Plasmodiophora brassicae* on *Lycopersicum esculentum* (Reuveni *et al*., 1985) and *Plasmodiophora brassicae* on Grapes (Boerner *et al.,* 1974). Since auxin content decreases in the infected host tissue due to the action of IAA oxidase IAA oxidase level in culture filtrate of *A. cepulae* and in the blight areas of Onion leaves (Annadurai & Motlag,1999).The culture filtrate obtained from the Ray's medium was purified and analyzed is presented in this paper.

MATERIALS AND METHODS

Crude enzyme preparation

Crude enzyme was obtained from the medium suggested by Ray (1956). The medium containing Glucose 20.0 g, Ammonium tartarate 9.2g, KH_2PO_4 5.8 g, $MgSO_4$ 2.5 g, $FeCl_3$ (1%) solution 0.6 ml, thiamine HCl 100 g, distilled water 1000 ml was taken in 5 litres Hoffkins flasks. The flasks containing medium were sterilized, cooled and inoculated with *Alternaria cepulae* and kept for incubation for 20 days at 32±1°C. After 20 days the culture medium was filtered through two layers of cheese cloth. The filtrate was centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatant was used as the crude enzyme preparation.

Estimation of IAA oxidase activity

IAA oxidase activity was estimated according to the method of Sequeria and Mineo (1966).The activity of IAA oxidase was determined by the rate of disappearance of IAA. The reaction mixture contained 0.25ml of 1mM DCP, 0.75ml of a mixture of 1mM IAA and 0.5mM $MnCl_2.H_2O$,3.5ml of 0.02 **M** (W/V) citrate buffer pH 5.5 and 0.5ml enzyme.The mixture was shaken and kept 1 hour for incubation at 32±1°C. After the incubation period was over 1ml of Salokowski reagent was added to 5ml of the incubation mixture. The pink colour developed was read at 530 nm in a shimadzu spectrophotometer. The control treatment was carried out in an identical manner except that the enzyme was added after the addition of Salokowski reagent.The unit of IAA oxidase enzyme was defined as µg of IAA destroyed per ml of enzyme in one hour (Imbert and Wilson 1972).

Estimation of Protein

The protein content was estimated according to the method of Lowry *et al.* (1951) using crystallilne bovine serum albumin as the reference protein. In the case of column chromatography, however the protein contents in the eluates from the column were estimated by measuring the absorbance at 280nm using shimadzu spectrophotometer.

Specific activity

Specific activity of IAA oxidase at different stages of purification was expressed as the unit of enzyme activity per mg of enzyme protein.

Estimation of IAA oxidase of *Alternaria cepulae* in culture medium

IAA oxidase activity was estimated from the culture filtrate of Ray's medium on different days with 4 days interval of time by adopting the method of Sequeira and Mineo (1966).

RESULTS

Purification

Ammonium sulphate precipitation

The culture filtrate obtained from Ray's medium after centrifugation was adjusted to pH 5.E and kept in a 2 litres beaker containing paddle for magnetic stirring at 4°C for 30 min. Solid ammonium sulphate was added slowly with gentle stirring to give 0-35%, 35-70% saturation. The precipitate formed was collected after centrifugation at 10,000rpm for 20 minutes. This was dialyzed against 0.02 M citrate buffer pH at 4°C for 48 hours.

Chromatographic Procedure

Then entire chromatographic operation were carried out at 4°C. Addition of sample to the column,elution of proteins and maintenance of column flow rate were performed using peristaltic pump (LKB model 2132 microperpex pump).The column effluents were continuously monitored at 230 nm using uv monitor (LKB uv cords II model 2238). The recording of absorbance was simultaneously done by LKB model 2210 recorder. Constant volume fractions were collected by a fraction collector (LKB redirac model 2112). Measurements of absorbance of collected fraction (A280) were carried out in a shimadzu spectrophotometer uv 260 Japan with 1 cm cuvette at 280nm.

CM Trisacryl column

The affinity chromatography column (2 X 29.5 CM) was packed with CM-Trisacryl (bed volume 2 X 16.5 CM). This was equilibrated with 0.02 **M** citrate buffer of pH 5.5. 150 ml of active fraction of 70% $(NH_4)_2\ SO_4$ fraction was loaded to the column. After the initial washing of the column with buffer, when the absorbance of the column effluents reached the base line elution of bound materials was carried out using 0.1**M** NaCl gradient. The column washed with 0.02**M** citrate buffer containing 1**M** NaCl and equilibrated with binding buffer after every chromatographic run.

Ultrogel Column

Further purification was carried out by gel filtration on ultrogel ACA 44. The column (1.2 X 105 CM) was equilibrated with 0.02 M citrate buffer pH 5.5 containing 0.1M NaCl. The IAA oxidase active fractions obtained from CM Trisacryl column were pooled, lyophilized in a virtis lyophilizer and dissolved and dialyzed solution was loaded to the ultrogel column and eluted with 0.02 M citrate buffer pH 5.5. The flow rate of the column was 5ml/hour and 4ml; fractions were collected in each tube at regular interval of time using the same eluting buffer.The tubes were directly read at 280nm for protein and analysed for IAA oxidase activity.

Tests for purity of the enzyme sample

The homogeneity of the purified IAA oxidase was determined by poly acrylamide disc gel electrophoresis, isoelectric focussing immunodiffusion and immunoelectrophoresis.

Polyacrylamide gel electrophoresis

It was carried out according to the method of Davis (1964). Acrylamide and Bisacrylamide solution 7.5ml, 3.8ml of separating or resolving gel buffer,water 15.3ml ,TEMED 20 µl and ammonium per sulphate were taken. The contents were mixed well and degassed using an aspirator and poured in to clean perpex tubes and allowed to polymerize. Usually 5-15 µl of purified enzyme samples (1mg/ml) dissolved in glycerol (1:0 v/v dilution) in water was carefully layered on the gel surface along with bromophenol blue as the tracking dye. The electrophoresis was conducted at 4°C. A starting current of 2mA per tube for 120 minutes was supplied across the electrodes. After the electrophoretic run, the gels were removed and stained for protein with the staining solution for 1 hour. Detaining was carried out destaining solution (10% v/v methanol and 7% V/V acetic acid in water) till the band appeared distinct.

SDS Polyacrylamide gelelectrophoresis

SDS PAGE was carried out with SDS Tris glycine system of Laemmli (1970). Enzyme protein (1mg/ml) dissolved in 1.25 **M.** Tris HCl buffer pH 6.8 containing 0.5 % each of SDS and β-mercaptoethanol was kept in a boiling water bath for 3 minutes in a tightly stoppered tube. 5-15 µl of this sample was loaded on the gel and the electrophoresis was conducted at a constant current of 4 mA per tube until the tracking dye reached the bottom. The staining and destaining were carried out.

Isoelectric focussing

Isoelectric focussing was carried out in a semi preparation manner using the polyacrylamide gel rods (11 X 165 mm) based on the method of Wrigley (1971) at 4°C. The gel was cast by mixing the following solutions. Acrylamide 30% W/V and 1% W/V N,N methylene bis acrylamide in water 4.0 ml, Ampholine (pH 3.5-10.0) 0.4ml, Enzyme protein 5mg, 1.0ml, water 9.6ml, Ammonium per sulphate (1%W/V) 1.0ml./ The anode and cathode solutions were 0.2% W/V phosphoric acid and 0.4W/V ethanolamine respectively. A constant power of 4 mv was applied for 2 hours using LKB model 2197 electro focusing power supply unit. The gel rod was then removed from the water jacked glass tube and the pH gradient generated during electrofocussing was assessed using a surface pH electrode.(LKB model 2117-111). The gels were then cut into 5 mm slices and the slices were shaken mechanically at 4°C with 1ml of glass distilled water overnight. Each fraction was measured for its pH and IAA activity respectively.

Immunological studies

Antiserum of the purified enzyme was prepared according to the method of Graber & Burtin, (1964). Adult white Rabbits (about 3 kg body weight) were given a course of six intra muscular injections each containing 3mg/ml enzyme protein in isotonic phosphate buffer of pH 7.2 emulsified with an equal volume of Freund's complete adjuvant at interval of 15 days. 25 days after the last injection blood samples were removed by ear vein puncture and allowed to clot for 1 hour. The antiserum was collected by centrifugation and stored in the deep freeze with a little methiolate.

Double Immunodiffusion technique

To study the homogeneity of IAA double immuno diffusion technique was carried out according to the method of Ouchterlony (1973). The antigen was diluted in the ratio of 1/2, 1/4,1/8,1/16 and 1/32 in PBS azide. 6µl of each of this diluted antigen was pipetted into peripheral well and the antisera were taken in the central wall. The slides were placed in a humid chamber and incubated for 48 hours at 4°C. The immunoprecipitin lines were examined in reflected light. The incubated slides were then washed with PBS for 1 hour and then rinsed in double distilled water; they were then stained in coomassie brilliant blue R 250 staining solution for 30 minutes and destained with destaining solution. After allowing the slides to dry the immuno precipitin lines were photographed.

Immunoelectrophoresis

Immuno electrophoresis was performed according to the method of Graber and Burtin (1964). 3 µl of antigen were loaded in to the wells on either side of trough. The electrophoresis was carried out at 6 V/cm. At the end of the run the slides were taken out and agar plugs carefully removed from the trough and rabbit antiserum was filled. It was kept overnight at 4*c for incubation. The immunoprecipitin arcs visible in reflected light were stained with coomassie brilliant blue R 250 and destained.

UV absorption

To 2ml of distilled water in a cuvette 20 ml of purified enzyme solution (1mg /ml) was added and the UV scan was done recorded between 190nm-340nm in a shimadzu spectrophotometer.

RESULTS

In ammonium sulphate precipitation of the culture filtrate of Rays medium, maximum IAA oxidase activity was recorded (Table 1) in 70% fraction which was further subjected to ion exchange column. When 70% ammonium sulphate fraction was eluted through CM Trisacryl column IAA oxidase activity was observed from 40th fraction to 64th fraction with a maximum amount of protein peak (Fig. 1).

TABLE-1: Ammonium sulphate precipitation of indoie acatic acid OXODASE (IAAO)

Sl. NO	Percentage $(NH_4)_2SO_4$	Volume(ml)	IAAO activity Units/ml	Protein content (µg/ml)	Spectific activity
1	Culture filtrate	900	88.8	61.35	1.45
2	35%	150	26.0	19.00	1.36
3	70%	190	367.8	28.00	13.13
4	100%	130	12.0	18.50	0.65

TABLE-2: Purification of Indole Acetic Acid Oxidase

Steps	Volume	Total protein (µg)(A)	Total IAAO acitity units (µg)(A)	Specific activity B/A	Purification Fold	Yieid (%)
Culture filtrate (Supernatany)	900	55220	79920	1.0	1.5	100.0
70% $(NH_4)_2SO_4$ Precipitation	190	5328	69890	13.1	9.1	87.5
CM-Trisacryl Column	140	890	59934	67.3	46.7	75.0
Ultrogel	95	380	46850	123.3	85.5	58.6

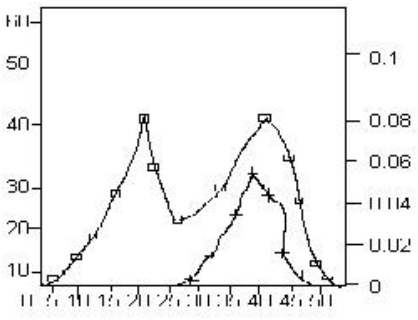

Figure 1: Elution profile of ultrogel column chromatography of IAA oxidase

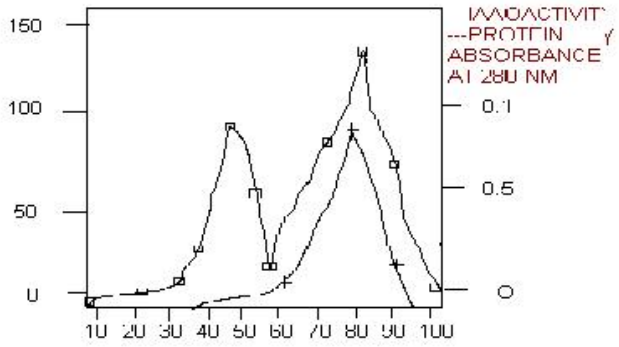

Figure 2: Elution profile of cm- trisacryl column chromatography of IAA oxidase

When this fraction was subjected to Ultrogel column (Fig. 2) protein activity was observed from 30 th fraction to 60th fraction but IAA oxidase activity in this fraction. But from 60th fraction to 92nd fraction IAA oxidase activity was the purification steps of IAA oxidase are given in Table 2. At each stage of purification, the enzyme solution was analyzed for specific activity. It is evident from the Table-2., that IAA oxidase was purified to 85.5 fold with the yield of 58.6% Both Polyacrylamide gel electrophoresis (Fig.3) and SDS polyacrylamide gel electrophoresis (Fig. 4) exibited single distinct band in coomassie blue staining of the purified enzyme. Results of isoelectric focussing in a semi preparative manner are shown in Fig.8&9. IAA oxidase activity is seen in 22nd and 23rd fractions and its

isoelectric pH is 5.0. The scanning of IAA oxidase for UV absorption between 190 nm to 350 nm is shown in Fig.2. It shows a single peak and the uv absorption is at 214.0 nm. Identity between both proteins is antiserum of IAA raised and pure enzyme IAAO was observed in Ouchterlony immunodiffusion (Fig.5) and immunoelectrophoresis (Fig.6).

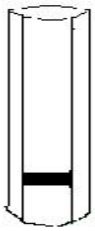

Figure 3: polyacrylamide disc gel electrophoresis of IAA oxidase

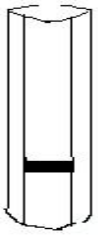

Figure 4: SDS- polyacrylamide gel electrophoresis of IAA oxidase

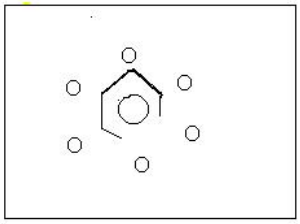

Figure 5: Ouchterlony's double diffusion Patterns of purified IAA oxidase

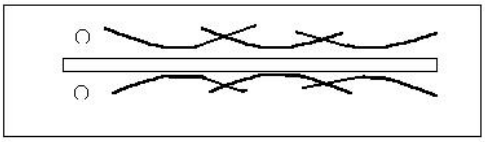

Figure 6: Graber & Burtin immunoelectrophoresis pattern of purified IAA oxidase

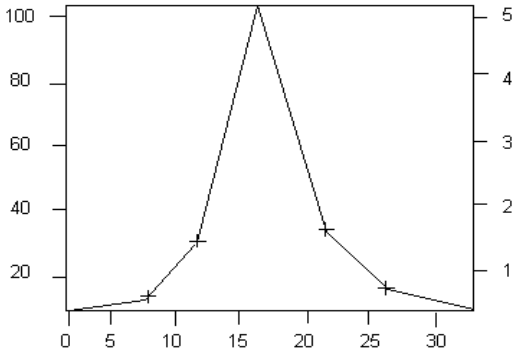

Figure 8: determination of isoelectric point of IAAO of *Alternaria Cepulae*

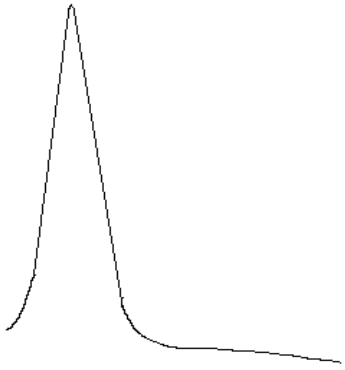

Figure: 9 UV Absorption Spectrum of IAAO

DISCUSSION

From the experiments conducted IAAO activity is maximum after the severity of the leafblight disease. The fact that micro organism produce IAA oxidase in infected plants by parasites is to influence the auxin concentration (Sequeira and Kelman,1962, Sequeira and Mineo,1962 and Mahadevan,1984.) From the results presented it is understood that IAAO activity was started only after the 20th day after inoculation. When the hyperauxiny is developed in the infection site,the need for IAAO to maintain the auxin concentration become necessary. Before the blight infection IAA was not present at the infection site,But when the disease become severe the translocation of IAA which stimulates the parasite to escape from the situation of hyperauxiny (Sequeria 1964,Mahadevan,1984.) Hence the production of IAAO from 16th day onwards is to degrade IAA concentration in the blight area.

IAA oxidase from tobacco roots was highly purified by Sequeria and Mineo,1966. However the crude enzyme IAAO was subjected sequentially to four different steps of treatment is to get purified enzyme with maximum yield and specific activity. When ammonium sulphate fraction was subjected to CM-Trisacryl column and eluted with 0.1m NaCl, the specific activity was found to increase upto 67.3 units with 75% yield. Fractionation of this product when applied to ultrogel column the specific activity increased up to 123.3 units with 58.6% yield. By this method an higher yield was obtained besides using SE-sephadex column (Sequria and Mineo,1966), Sephadex G 100 column (Meudt,1972),CM Sephadex column (Miyata,1981).

Presence of multiple forms of the enzyme was not observed during the purification. The purified enzyme

migrated as a single band in ploy acrylamide gel electrophoresis and SDS-PAGE. Iso electric focussing showed a single peak at iso electric pH5.0. The immuno diffusion shows that not more than one antigen is present in this system, the partial identity indicates that some differences may be there in the tri dimensional structure of two proteins. The immunopreciptin arcs observed in the Immuoelectrophoresis, antigen and antibody interaction occurred showing the homogeneity of the enzyme.

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