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Carboxylesterases of Sesbania Grandiflora Seeds

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ABSTRACT: Sesbania grandiflora seeds were selected to study carboxylesterase. Germination studies were carried for 178 hours to know the enzyme's activity at different stages of initial germination process. Activity was found maximum on the third day. Carboxylesterases were partially purified from the germinating seeds using conventional chromatography techniques like ammonium sulphate precipitation, ion exchange chromatography, PAGE. The specific activity and yield of the enzyme were found to be 1559U/mg and 6 respectively. Three different isozymes were found in SDS-PAGE studies with molecular weight 28, 25 and 22 kDa.

KEYWORDS: Sesbania grandiflora, Carboxylesterases, Germination, SDS-PAGE, Isozymes, Defense Mechanism, Herbicide detoxification, and Molecular weight.

I. INTRODUCTION

In enzymology, a carboxylesterase (EC 3.1.1.1), is an enzyme that catalyzes the chemical reaction.



Carboxylesterases are important for the metabolism of plant growth and development. Plants are frequently exposed to natural and synthetic toxins like pesticides and pollutants. Consequently plants must have specific and coordinated defence mechanism for their survival. Carboxylesterase is found to be one of the enzymes involved in herbicide and safener metabolism. (Dean E. Riechers et.al., 2010). They also play a significant role in agrochemical efficacy and detoxification by interacting with three major classes of agrochemicals OPs, carbamates and pyrethroids. Studies have shown that variations in the carboxylesterase levels and its isozyme abundance play a significant role in the relative toxicity of ester containing insecticides in a range of organisms from insects to animals. Even in the humans carboxylesterases play an important role in hydrolyzing of some drugs like coca ethylene (S. Casey Laizure et.al., 2003), Clopidogrel which is an antiplatelet drug (John R. Horn and Philip D. Hansten, 2013). In view of the significance of carboxylesterases and importance of Sesbania grandiflora with reference to its medicinal values which include antioxidant and cytotoxic activities, and its extensive use in folk and traditional medicine the present investigation was carried out to study the variation in esterase activity during the germination of Sesbania grandiflora seeds and to purify and characterize the enzyme. The present project gives an insight of the carboxylesterases in these germinating seeds. So far no studies on esterases in Sesbania grandiflora have been carried out; hence in view of its significance it is interesting to carry out this study.

II. MATERIALS AND METHODS

Sesbania grandiflora seeds were collected from IIHR Bangalore. The plant has various uses in folk and traditional medicines for headache, swellings, anemia, bronchitis, pains, liver disorders and tumors, CNS depressant, hypertensive, cardiac depressant, diuretic, anti-inflammatory, antibacterial, antipyretic, analgesic, hypoglycemic and cytotoxic activity.

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Germination studies: Healthy *Sesbania grandiflora* seeds were selected, washed, soaked in distilled water and allowed to germinate. The samples were collected every 24 hours and the enzyme activity according to Gomori (1953) and Van Asperen (1962) and estimation of protein content by Lowry et al method (1951) was carried out for up to 178 hours.

Extraction of Esterases: The above germinating seeds at different stages were used for enzyme extraction. Acetone powder was prepared according to the method given by Wetter (1957). From this acetone powder, 10% enzyme extract was prepared in ice cold conditions using 0.05 M phosphate buffer. Estimation of the protein content and enzyme activity was carried out in the crude by the above mentioned method for the germination studies.

Enzyme purification: The crude was further subjected to ammonium sulphate precipitation. 40ml of sample was taken and 0-30% cut was given with ammonium sulphate (7.04g/40ml). Finely powered ammonium sulphate was added to the sample slowly with stirring on an ice bath and was incubated at 4°C for 10 minutes. The obtained precipitate was removed by centrifugation at 10,000rpm, 4°C for 30mins. The supernatant obtained, was taken and 30-80% cut was given with ammonium sulphate (14.596g/41ml). Finely powered ammonium sulphate was added to the sample slowly with constant stirring on an ice bath and was incubated for 10 minutes. Centrifuged to separate precipitated proteins at 10,000rpm, 4°C for 30mins. The pellet was resuspended in minimum volume of 50mM Sodium Acetate Buffer pH-5.4. The suspended pellet was dialysed against 2 litres of 50mM sodium acetate buffer pH 5.4, with 3 changes at 4°C. The dialysed pellet was centrifuged at 10,000rpm for 20mins at 4°C. The supernatant was measured as 26ml which was loaded to column.

Ion exchange chromatography: The dialysed ammonium sulphate precipitate was allowed to pass through the column at the rate of 30ml/hour. Unbound proteins coming out of the column is collected in a tube (Breakthrough). Wash buffer was passed through the column, and collected 10ml fractions and continued wash till the A_{280} of the wash falls below 0.05. The bound proteins were eluted from the column using 60 ml each of elution buffer. 6 ml fractions were collected. The A_{280nm} of the fractions were checked. This is the eluate. Before storing the column at 4°C, the column was washed with 100ml of regeneration buffer.

PAGE: A native page was carried out using polyacrylamide gel 12% followed by staining of esterases according to Hunter and Markert method (1957). Proteins were detected on polyacrylamide gels using 0.5% solution of coomassie brilliant blue R-250 in 25% methanol and 7.5% acetic acid in water.

SDS-PAGE: Determination of molecular weight carried out by SDS- PAGE. When the dye front comes to 0.5 cm above the bottom of the gel, power pack was turned off. The plates were carefully separated apart and the gel was rinsed with distilled water. The enzymes were renatured by using Triton X100 and incubated on a rocker for 30 minutes, rinsed with sodium phosphate buffer pH 7.2 and the bands were developed by the above mentioned method for PAGE.

III.RESULTS

Time in hours		12	24	48	72	98	120	146	178
	Dry seeds								
Activity U/ml	1704(±0.1)	1800(±.03)	1813(±.04)	2186	2560(±.01)	1040(±.02)	2240(±.02)	426(±.0.2)	1333(±.01)
Protein mg/ml	15.00(±.1)	17.10(±.2)	18.50(±.3)	19.50	20.00(±.02)	10.50(±.02)	19.00(±.04)	8.65(±.1)	13.00(±.01)

Table -1. Enzyme activity and protein content of soaked and germinating seeds at a variety of time duration

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Germination studies of soaked seeds show variation in the activity and protein content at different time period. There is a gradual increase in the activity for up to 72 hours and a decrease at 98 hours and 146 hours. The activity is found show an increase at 120 hours and 178 hours.

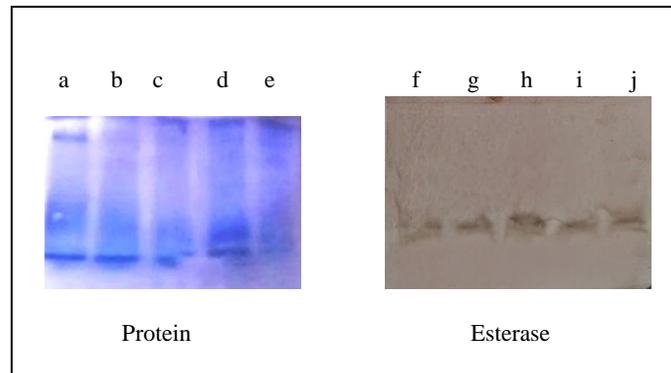


Fig.1. Native PAGE pattern of protein amount and enzyme activity from dry seeds to 78 hour germinating seeds.

A native PAGE was run by loading 20 μ l of the enzyme extract. The intensity of the bands developed support the results obtained from Spectrophotometric assay as shown in Fig.1 and Fig.2.

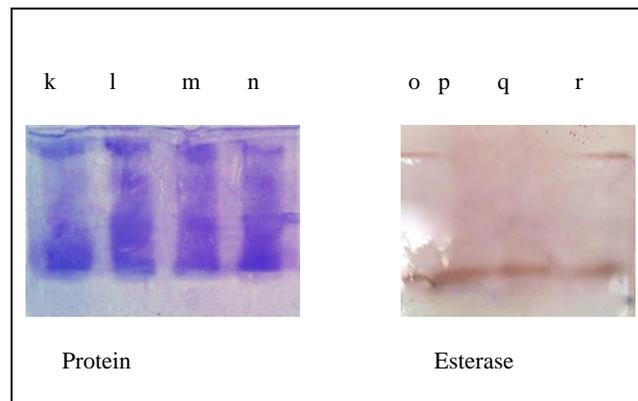


Fig. 2. The electrophoretic pattern of protein content and esterases from 98 hours to 178 hours germination period.

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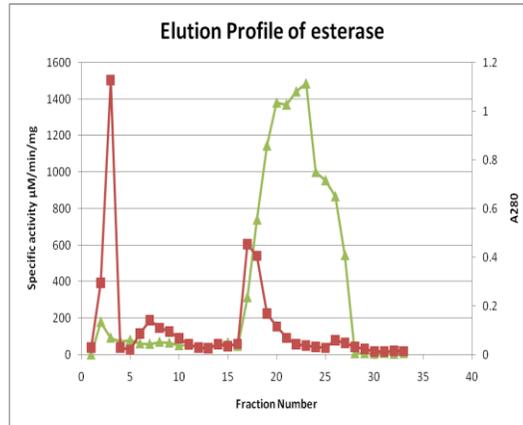


Fig. 3. The elution profile of esterases from soaked seeds of Sesbania grandiflora on CM Cellulose.

The eluate obtained from CM Cellulose column was subjected to quantitative and qualitative estimation of protein and esterases. Each time 6 ml fraction was collected. The specific activity was found to be maximum at 23rd fraction. The overall purification scheme is summarized in Table 2. Specific activity of esterases is found to be 1559U/mg.

Purification step	Activity U/ml	Protein U/ml	Specific activity(U/mg)	Fold purification	% yield
Crude	1038.36(±0.1)	26.68±1	38.91	1	100
Ammonium sulphate precipitation	440(±2)	3(±.1)	146.6	3.76	42.37
CM-Cellulose Fraction	62.3(±1)	.04(±.001)	1559	40.06	6

Table-2 Purification steps of esterase from Sesbania Grandiflora seeds

PAGE



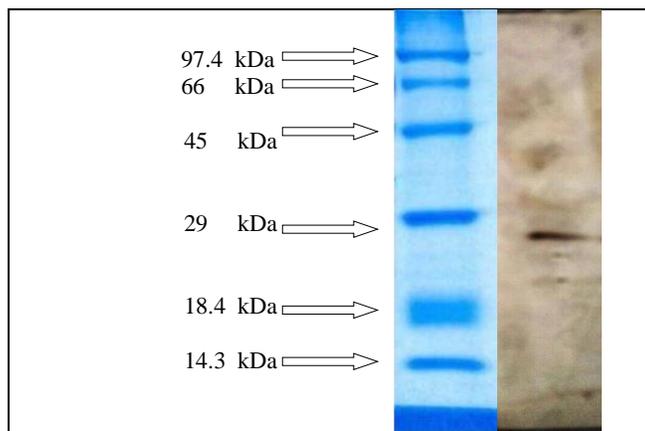
Pic-5.Bands stained for esterases.

A polyacrylamide gel electrophoresis was carried out by loading 20µl of eluate obtained from CM-Cellulose fractionation. The result shows two bands followed by the staining the gel for esterases.

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Pic-6.SDS-PAGE

Molecular weights were determined by 12% SDS-PAGE. The gel was cut into two halves, the first half gel loaded with protein marker was stained with coomassie brilliant blue R-250 and the other half was treated with Triton X for 30 minutes allowing the proteins to renature and the gel was stained for esterases. Three bands were developed and the molecular weight was found to be 28, 25 and 22kDa.

IV.CONCLUSION

Present studies in germination of *Sesbania grandiflora* seeds show a lot of variation in the activity of the enzyme at different stages and the maximum activity was found at 72 hour. Similar kind of variations in the enzyme activity during germination has been reported in Lettuce seeds (G.Ram chandra and Vivian K.Toole, 1977) and *Tamarindus indica* (S.Kantharaju, K.R. Sidhalinga Murthy, 2014). Our findings of molecular weight of esterases in *Sesbania grandiflora* indicate that they are in the low molecular weight range. R.Stoubman et.al., have shown two isozymes in *Jatropha curcas* seeds whose molecular weight is 21.6-23.5, and 30.2kDa. In *Macuna Pruriens* seeds investigated by K.S.Chandrashekariah, the molecular weights of esterase isozymes were found to be 20.89 and 31.62kDa. Similar findings were observed in *Cesalphina mimosidae* seeds (Bhavita.K.P. et.al., 2014), where the molecular weight is reported to be 20kDa. Thus our findings in *Sesbania grandiflora* support the earlier research literature. The importance of these enzymes in metabolism of agrochemicals and insect resistance is well established. The enzyme is also being used in designing soft and prodrugs for the pharmaceutical market as well as the use of esterases activity in development of anti-tumor agent CPT-11. Taking into account the significance of esterases and the medicinal value of *Sesbania grandiflora* it is worth to investigate esterases as there are no such studies so far in this species. Further characterization of the enzyme is in progress.

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