Partial purification and characterization of esterase from the seeds of *Leucaena leucocephala*

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Abstract
Esterase plays a pivotal role in the biochemical mechanism during germination and associated with multiple aspects of plant physiology and developments. In this research work, esterase was purified to homogeneity with 7.9 fold by a four-step procedure comprising crude extract, ammonium sulphate fractionation, CM-Cellulose and Sephadex G-150 chromatography from 3rd day germinated endosperm of Leucaena leucocephala. Molecular weight of partially purified enzyme is 29 kDa approximately and determined by SDS-PAGE and gel-filtration chromatography. The enzyme was fairly stable at moderate temperature and neutral pH conditions.

The specific activity and yield percentage of the enzyme were found to be 0.11µmoles/min/mg and 11.37 respectively. The Km and V_{max} were 0.10mM and 7.14 nmoles /min respectively. The enzyme shows inhibitions with Dichlorovos and IC_{50} was 1×10^{-6} M.

Keywords: Esterase, germinate, purification, yield.

Introduction

Esterase EC.3.1.1.1(carboxyl ester hydrolase) is hydrolytic enzyme which can catalyze the hydrolysis of various types of esters bonds especially short chain of aliphatic and aromatic carboxylic ester compounds. This enzyme is plentiful in plants, animals and microorganisms. In plant, this enzyme plays role in development and metabolic process. Esterase is useful in degrading natural and industrial waste which includes cereal waste, plastics and other poisonous materials[7]. Various industries use esterase for cosmetics, paper and pulp, food processing, detergent constituents, synthesis of carbohydrate derivatives and food additives to increase the flavour etc. It plays an important role in endobiotic processing of hydrophobic compounds like cholesterol[8]. The global market for the enzyme is constantly growing with a rate of 5-10 % per year.

Leucaena leucocephala is also known as river tamarind or subabul and presently spread across the world. It is one among 100 invasive species listed in International Union for Conservation of Nature (IUCN). This plant grows very quickly in arid areas with a wide range of soil pH. This leguminous tree has various uses like fuel woods, fodder, shade, paper pulp, biofuel, worm repellent and green manure. Seeds of *L.leucocephala* are rich in antioxidants, phenol, proteins and various soluble and insoluble polysaccharides. It has anti-nutritional factors like oxalate, alkaloid, phenol, tannin, saponin and phytate. Seeds of this plant possess mimosine, a non-protein compound chemically similar to dihydroxyphenylalanine with a 3-hydroxy-4-pyridone ring instead of a 3, 4-dihydroxy phenyl ring. In this present study, seed is found to be promising source of esterase enzyme.

Material and Methods

Materials: Dry seeds of *L. leucocephala* (river tamarind) were collected from Krishna- Gowda Park near Basavangudi, Bangalore, Karnataka, India. Seeds are surface sterilized using $KMnO_4$, dried at 40- 45 ºC for 5-6 h and stored in an airtight container at 4 ºC for future use [9].

Chemicals: Acetic acid, acrylamide, ammonium per sulphate, CM-cellulose, Fast blue RR salt, FC Reagent, BSA, Coomassie Brilliant Blue R-250, methanol, 1-naphthyl acetate, N, N methylene bis-acrylamide, β-mercaptoethanol, sodium chloride, Sephadex G-150 were obtained from SRL Chemicals.

Seed germination: Seeds were soaked for 48 h in distilled water. The imbibed seeds were set to germinate for 10 days at room temperature (28 °C ±2) in sterile Petri dish lined with five to six layer of filter paper. Seeds were supplied with Hoagland media no. 2. On 3rd day, endosperms were harvested, dehulled and used for enzyme purification and kinetic studies.

Crude enzyme extraction: 10% extraction (1g/ 10 ml) of the endosperm was prepared in chilled phosphate buffer (30 mM pH 7) by homogenizing in a pestle and mortar for 10 min and centrifuged at 7,000 rpm for 15 min at 4˚C. Pellet was discarded and the supernatant was used for the assay.

Purification of enzymes

A) Ammonium sulphate fractionation: To the crude extract, finely powdered ammonium per sulphate was added to 0–40% saturation and the obtained precipitate was removed by centrifugation at 7,000 rpm for 10 min at 4˚C. For the supernatant obtained, finely powdered ammonium per sulphate was added to get 40–80% saturation at 4 ˚C. The protein was precipitated by centrifugation at 7,000 rpm for 10 min. The precipitate thus obtained was dissolved in 30 mM sodium phosphate buffer, pH 7 and dialyzed against the 6 mM sodium phosphate buffer, pH 7 for 24 h at 4 ˚C.

B) Ion Exchange chromatography: The dialyzed sample was loaded onto a CM-cellulose column (1x50 cm) pre-equilibrated with assay buffer. 5ml fractions were collected

at a flow rate of 30 ml/h. The bound proteins were eluted with stepwise increase in concentration of sodium chloride. For all fractions, assay for protein and esterase activity was performed.

C) Gel filtration chromatography: Sephadex G-150 matrix pre-equilibrated with assay buffer in 1x50 cm column. 1ml fractions were collected at a flow rate of 7 ml /h. For all fractions, assay for protein and esterase activity was performed.

Gel Electrophoresis: Native PAGE (10 %) was performed at pH 8.3. The electrophoresis was performed at 4 ˚C for 2 h. After the run, gels were incubated in assay buffer for 20 min and decanted followed by staining for activity and protein. SDS-PAGE (10%) was performed after denaturing the proteins with SDS and β-mercaptoethanol. Gels were stained for protein.

Staining of gels- Activity Staining: The gels were stained for esterase activity with 50 ml of 0.05 M phosphate buffer pH 7.0 (5 mg of fast-blue RR and 20 mg of 1-naphthyl acetate dissolved in 1 ml of acetone) for 20 min at room temperature.

Protein staining: The gels were stained for proteins using 0.01% Coomassie brilliant blue R-250 and destained in methanol and acetic acid (9:1).

Enzyme Assay: Esterase activity was assayed according to Bhavith et al[1] with slight modification. The reaction mixture was consisting of 5 ml substrate (0.50 mM α napthyl acetate in 30 mM phosphate buffer pH 7) and 1 ml enzyme extract (suitable dilution) and incubated at 37 ºC for 20 min. The reaction was stopped by the addition of 1 ml of DBLS reagent (2 parts of 1% diazo blue B and 5 parts of 5% sodium lauryl sulphate).

Control was performed by adding DBLS to the enzyme before substrate for enzyme inactivation. The absorbance was measured at 600nm against blank. Total enzyme activity was measured taking α napthol as standard. Total protein content was estimated according to Lowry et al[10], using BSA (bovine serum albumin) as standard. The protein content in the fraction obtained from chromatographic columns was measured at 280nm.

Kinetic studies

(a) Effect on time: Partially purified esterase was incubated with substrate for (1, 3, 5, 8, 12, 20, 30min) at room temperature. The assay for the enzyme was performed as mentioned above.

(b) Effect of substrate concentration: Partially purified esterase was incubated with different concentration of substrate (0.03, 0.05, 0.07, 0.08, 0.10, 0.13, 0.17, 0.2 µmoles/ml) and the product obtained was measured using spectrophotometer at 600nm.

(c) Effect of pH: Partially purified esterase was studied with different pH (4.0, 4.5, 5.0, 5.5 pH of acetate buffer), (6.0, 6.5, 7.0, 7.5, 8.0 pH of phosphate buffer), (8.0, 8.5, 9.0 pH of tris HCl buffer) and the product obtained was measured using spectrophotometer at 600 nm. pH stability was performed by incubating the enzyme with above mentioned pH buffer for 20 min and the assay was carried out.

(d) Effect of temperature: Partially purified esterase was incubated with substrate at different temperature (7, 15, 22, 28, 30, 37, 45, 52, 60, 70 °C) and the product obtained was measured using spectrophotometer at 660 nm. Temperature stability was performed by incubating the enzyme with above mentioned temperature for 20 min and the assay was carried out.

(e) Effect on inhibitors: Partially purified esterase was allowed to pre-incubate with different concentrations of inhibitor (Dichlorovos) 1×10^{-2} - 1×10^{-10} M and assay for the activity was carried out as mentioned above. Percentage of inhibition was calculated for each concentration with the equation mentioned below:

$$\% \text{ of inhibition} = \text{control} - \text{A } 600\text{nm} / \text{control}$$

Results and Discussion

The leguminous plant *L. Leucocephala* is widely grown plant enriched with various bioactive compounds, antioxidant, phenolics, polysaccharides etc. It is easily available protein source and consumed by both animals and human. Seeds are a rich source of esterase enzyme which has established usefulness in various fields like industry, medical, protein engineering and pro-drug synthesis.

Germination of *L. Leucocephala* seeds with cocopit sand mixture (1:1) shows fungal growth. Seeds germinate in filter paper layer supplemented with Hoagland Media no. 2 without any contamination. During germination on 3rd day, total activity for esterase was maximum and selected for further purification. The differential fractionation with chilled acetone and pH results in inactivation of esterase.

However, ammonium sulphate fractionation shows significant activity and was chosen for further purification. The binding affinity of protein to cation exchanger (CM-Cellulose) indicates the presence of a large portion of basic amino acids[1].

Table 1 summarises the isolation process, percentage of yield and purity of esterase obtained from *L. leucocephala.* Figure 1 shows elution profile of esterase from 3rd day germinated seeds of *L. Leucocephala* on CM- Cellulose. Proteins were eluted with stepwise increase in sodium chloride concentration. Figure 2 shows elution profile of esterase on Sephadex G-150 with pooled fraction obtained from ion-exchange chromatography.

Table 1
Purification table of esterase enzyme

Purification steps	Total activity (µmoles/min/g)	Total protein (mg/g)	Specific activity (µmoles/min/mg)	Percentage yield	Fold purification
Crude	3.47	234.23	0.0148	100.00	1.00
Ammonium sulphate	0.4457	60.88	0.0073	12.84	0.49
CM-Cellulose	0.6984	13.34	0.0523	20.12	3.53
Sephadex G-150	0.3947	3.36	0.1170	11.37	7.93

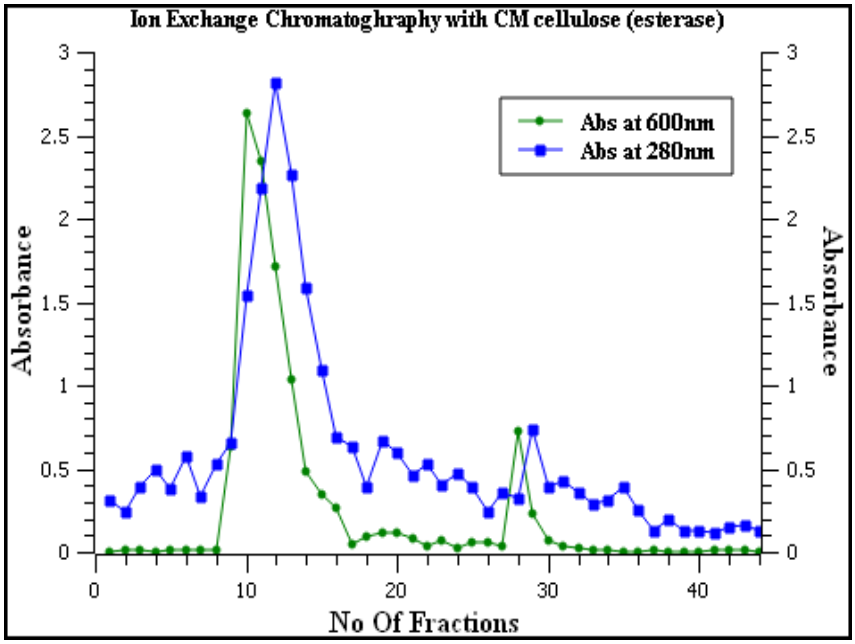


Figure 1: Elution profile of esterase from 3rd day germinated seeds of *L. Leucocephala* on CM- Cellulose. Proteins were eluted with stepwise increase in sodium chloride concentration.

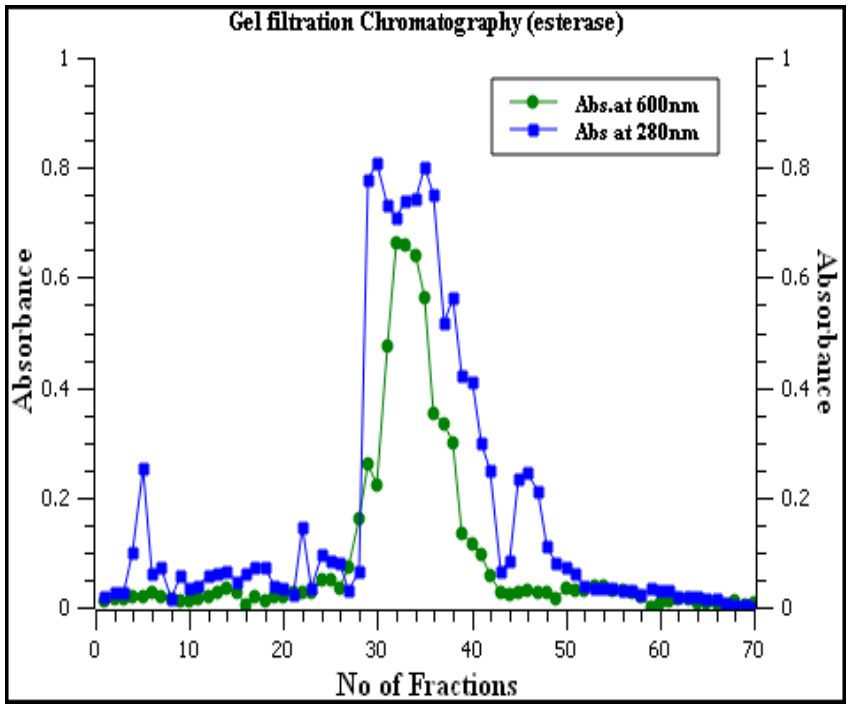
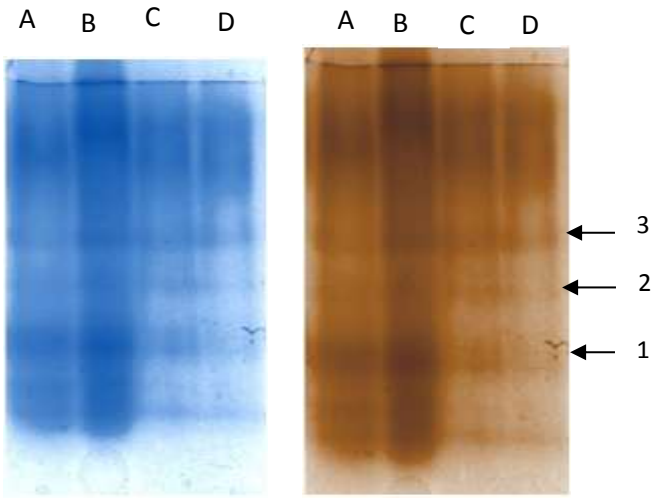


Figure 2: Elution profile of esterase on Sephadex G-150 with pooled fraction obtained from ion-exchange chromatography.



(a) **(b)**

Figure 3: Native PAGE (a) protein staining (b) Activity staining A. crude B. Ammonium precipitation fraction C. Ion-exchange fraction (pooled fraction 1 and 2) D. Gel filtration fraction

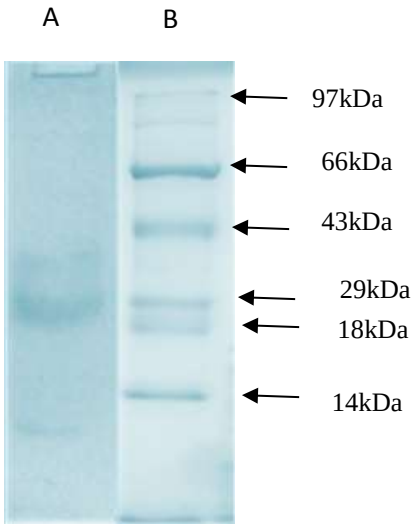


Figure 4: SDS PAGE A. Gel filtration fraction B. Molecular markers

Electrophoretic analysis of different methods of purification for esterase shows 3 bands (Figure 3a and 3b) among which band 1 is major. Three bands were obtained in the presence of β- mercaptoethanol (Figure 4) indicating that purified protein may comprise of three polypeptide chain. A plot of absorbance against time indicates that the reaction was linear up to 20 min (Figure 5a). The Km and V_{max} were 0.10mM of α-napthyl acetate and 7.14 nmoles /min respectively (Figure 5b). Partially purified esterase showed that the enzyme is stable at 37 °C and pH 7 (Figure 5c and 5d) respectively. Inhibition studies with dichlorvos an organophosphate insecticide shows IC_{50} 1×10^{-6} mM (Figure 5e).

Among plants, the first carboxylesterase was reported from peas in 1968. Apart from that is was also reported in barley, green beans, leaves of *Festuca pratensis*, apple, the latex of *S. grantii* and finger millets[3]. Source of this enzyme includes microorganism (bacteria /fungi), yeast, plants and animals. Esterase can breakdown the long chain of fatty acid. There are about 350 unique type of esterases which were identified when they were coupled with chromophore[4]. Esterase obtained from *Cynara Cardunculus L.* and *Ficus Carica L.* is important to release odorous compounds in cheese making[2].

Nowadays, due to versatile uses esterase is gaining interest in research. Esterase is used in the treatment of several types of cancer like lung cancer, colon cancer and hereditary angioedema (HAE).

High expression of esterase is seen in tumours of lung colon and liver which can be exploited for selective drug conversion using ester pro-drug strategy[6]. As esterase has so many industrial uses like in cosmetics, detergent composition, synthesis of carbohydrate derivatives, food additives etc. which are ever increasing. To meet demand, this non-traditional river tamarind seed is very much potent[5].

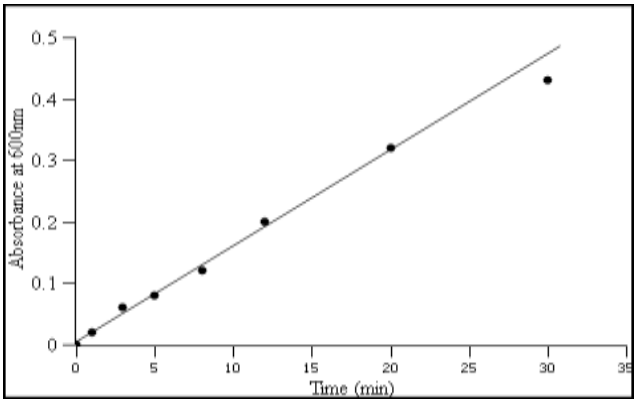


Figure 5(a): Effect of time on the activity of partially purified *Leucaena leucocephala* seed esterase

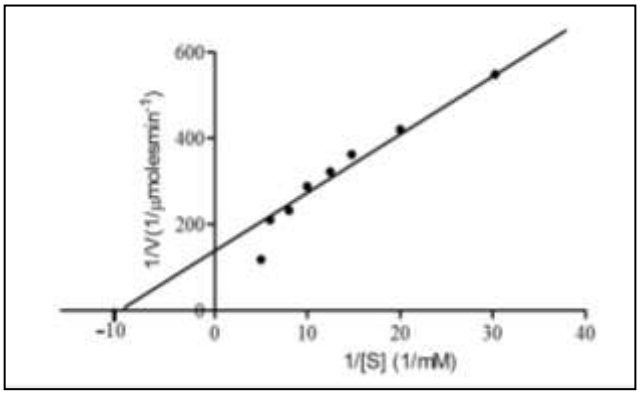


Figure 5(b): LB-plot of partially purified *L. leucocephala* seed esterase

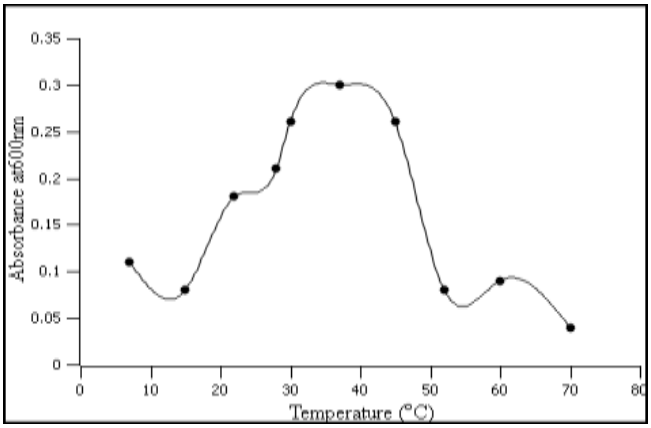


Figure 5(c): Effect of temperature partially purified *L. leucocephala* seed esterase

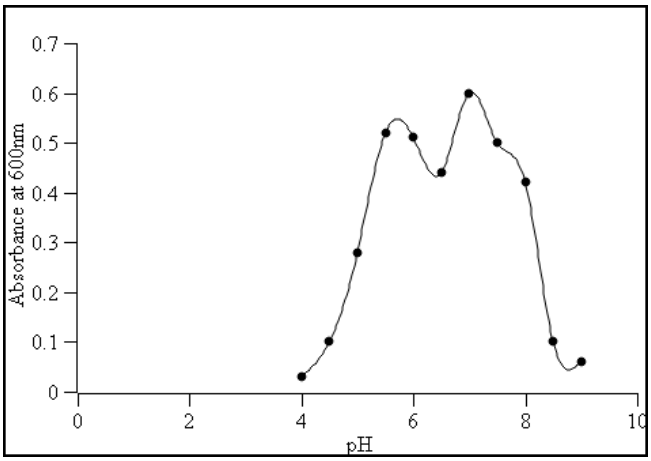


Figure 5(d): Effect of pH on partially purified *L. leucocephala* seed esterase

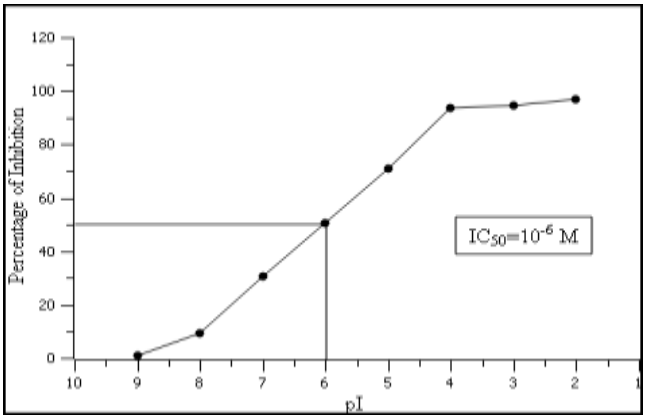


Figure 5 (e): Effect of Dichlorovos on partially purified *L. leucocephala* seed esterase

The esterase obtained from *Leucaena leucocephala* was found to have a stability range of 22-37ºC and 6-7.5 pH respectively. As this enzyme is stable at room temperature with neutral pH commercial isolation and purification is quite convenient.

Conclusion

In our study, we have chosen *L. leucocephala* seeds which are usually grown like weeds and have not attracted many people. Aim of this study is to promote unusual sources of the useful enzyme to meet the ever-increasing demands.

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