Physico-Chemical Properties of Purified Carboxylesterase from the Seeds of *Tamarindus indica*

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Authors' contributions

Both the authors have equal contributions in designing, executing and preparation of manuscript. Both authors read and approved the final manuscript

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ABSTRACT

The present work is focus on physical and chemical properties of purified Carboxylesterase using the Seeds of Tamarindus Indica. The esterases are extracted from the germinating tamarind seeds using 50 mM phosphate buffer, pH 7 and purified. The *Km* with *α*-naphthyl acetate, β-naphthyl acetate and *α*-naphthyl butyrate as the substrates is 28.6 µM, 22.2 µM and 26.7 µM respectively. The Vmax for the same substrates is 7.1×10^{-3} µmole/min, 7.41×10^{-3} µmole/min and 8.00×10^{-3} µmole/min respectively. The enzymes optimally active at pH 7.0 and are stable between pH 5.0 to 8.0. The optimum temperature of esterase activity is 40°C. The molecular weight of 27.5 kD as determined by SDS-PAGE, both in the presence and absence of β-mercaptothanol and is in close agreement with the molecular weight determined by gel-filtration on Sephadex G-100 (26.9 kD).

Keywords: Tamarind; carboxylesterase; gel filtration; saphadex G-100; SDS-PAGE.

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1. INTRODUCTION

Carboxylesterases are highly efficient catalysts that hydrolyze a wide range of aliphatic and aromatic esters, including amides and thioesters. They are important in the metabolism of drug and pesticides [1-11] are involved in the transfer of the acyl group to the accepters other than water. Carboxylesterases from the liver have been studied most extensively and molecular and catalytic properties of the purified enzymes have been thoroughly investigated. Xenobiotic-hydrolysing carboxylesterases are described in plants using model substrates such as α-naphthyl acetate. Although the old studies did not ascribe specific functions to these enzymes, major differences in esterase isoenzyme content between plant species and even within cultivars were demonstrated [12]. The hydrolysis of drugs and pesticides by esterases radically alters biological activity and trans-membrane transport. The respective hydrolases are key proteins in determining bioavailability and bioactivity [13]. In microorganisms, the hydrolases active toward carboxylesters, thioesters and synthetic amides, have been studied in some detail due to their importance in the biodeterioration of polymers and the bioremediation of pollutants [14]. In mammals and insects, these enzymes have attracted attention due to their roles in drug and insecticide metabolism, respectively [15].

In contrast, the corresponding enzymes in plants have received far less attention, even though they are important in determining the uptake and biological activity of important classes of herbicides, fungicides and insecticides [16]. For example, insecticides such as the pyrethroids are detoxified by plant esterases, limiting their bioavailability in crop protection [17]. Esterases also bioactivate pro-herbicides to their phytotoxic alcohols or acids, with the differential rates of hydrolysis in different plants contributing to selective weed control [18]. The relationship between chemical structure and the rates of pesticide hydrolysis in plants would be a useful tool in agro chemistry for predicting the relative rates of pesticide detoxification / bio activation in crops and weeds. A greater knowledge of these enzymes would also be useful in determining their largely uncharacterized roles in plant secondary metabolism. Currently, porcine liver esterase (PLE) is used as a model hydrolase to predict the likely rates of cleavage of synthetic esters in both the pharmaceutical and agrochemical industries. While this model enzyme may usefully reflect the activities of drug metabolizing hydrolases in animals, it's utility in accurately predicting the activity of esterases in plants has not been reported. It would be useful to identify model esterases in plants and compare their structure-activity profiles. Pesticide-detoxifying enzymes such as carboxylesterases (CXE, EC 3.1.1.1) make the pesticide ineffective against individuals that naturally have high levels of these enzymes [19]. These esterases detoxify organophosphate (OP) and carbamate pesticides and synthetic pyrethroids by two main ways - hydrolysis of the ester bond and binding of the pesticide (OPs) to the active site [20-22].

The studies on the properties of the purified carboxylesterases of plant seeds are scanty, on account of the difficulties involved in the purification. Esterase from seeds of Jatropha curcas has been purified to homogeneity and characterized as a carboxylesterase [23]. Similarly, two esterases have been purified from Mucuna pruriens and characterized as carboxylesterases [24]. Esterases have a definite role to play in plant growth and development, cell wall expansion, somatic embryogenesis (as a cytochemical marker) stomatal movement, insecticidal resistance against infection as a bioassay for environmental quality and secondary metabolic processes (hydrolysis of pyrethroid insecticides, cardenolides transformation and metabolism of tropane alkaloids, Indole alkaloids monoterpenoids and triterpenoids) [25]. In the present study, esterase purified from 21 days old germinated seeds of tamarind has been characterized as a carboxylesterase on the basis of substrate specificity in conjunction with inhibitor specificity.

2. MATERIALS AND METHODS

2.1 Materials

α-naphthyl acetate, α-naphthyl butyrate, β-naphthyl acetate and α-naphthyl phosphate, and all other chemicals used were of analytical grade. Dichlorvos, eserine sulphate, PCMB, phosphorylase b (94 kDa), bovine serum albumin (66 kDa), Ovalbumin (45 kDa), carbonic anhydrase (29 kDa), soyabean Trypsin inhibitor (20.1 kDa), lysozyme (14.3 kDa), cytochrome c (12.2 kDa) and Blue dextran (2,000 k Da) were purchased form Sigma Aldrich, India.

2.2 Methods

2.2.1 Determination of molecular weight by SDS–PAGE

SDS-PAGE of the purified esterase is carried out both in the presence and absence of β mercaptoethanol [26]. The 10 % SDS gels are inserted into the electrophoretic apparatus. The SDS treated purified esterase and standard samples (bovine serum albumin 66 kDa, carbonic anhydrase 29 kDa, trypsin inhibitor 20.1 kDa and cytochrome c 12.2 kDa) are loaded onto different wells along with 20 µl of glycerol and 2 µl of bromophenol blue. The electrode chambers are filled with reservoir buffer (14.3 g glycine, 3.4 g tris and 1 g of SDS in 1 litre of water, pH 8.3). The proteins are subjected to electrophoresis for 1.5 hours (till the bromophenol dye reached the bottom). The gel is removed and the protein stained using staining solution (0.5 g Coomassie brilliant blue R-250, 45 ml methanol, 7.5 ml glacial acetic acid and dilute to100 ml) for 1 hr. The gel is destained using the destaining solution (25 ml methanol, 75 ml glacial acetic acid and 67.5 ml water) and stored in 7 % acetic acid.

2.2.2 Determination of molecular weight by gel filtration on sephadex G-100

The gel-filtration using 117 ml of Sephadex G-100 column (1.22 cm X 100 cm) is carried out. The marker proteins (1-2 mg) and purified esterase (1 mg) are (dissolved in 50 mM, phosphate buffer pH, 7.0) loaded onto the column separately and are eluted with the same buffer at a flow rate of 10 ml / hr. Fractions of 2 ml are collected. The marker proteins used are phosphorylase b (94,kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soyabean trypsin inhibitor (20.1 kDa) and lysozyme (14.3 kDa). Blue dextran (2,000 kDa) is used to determine the void volume (V_o). The marker proteins and the purified esterase are detected by measuring absorbance at 280 nm for protein and that of esterase by determining the activity with α-naphthyl acetate, respectively.

3. KINETIC ANALYSIS

3.1 Effect of Time

Effect of time on the purified carboxylesterase from the seeds of tamarind is determined by assaying the purified carboxylesterase using α-naphthyl acetate for different time intervals.

3.2 Effect of Enzyme Concentration

The rate of the reaction is determined by assaying different concentrations of the purified enzyme (2, 4, 6, 8, and 10 µg) using α-naphthyl acetate for 15 mins.

3.3 Effect of Substrate Concentration

The substrate specificity of the purified carboxylesterase is determined by assaying the purified enzyme using different ester substrates, such as, α-naphthyl acetate, α-naphthyl butyrate, β-naphthyl acetate and α-naphthyl phosphate for 15 mins. The absorbency of α-naphthol and β-naphthol are measured at 600 nm and 540 nm, respectively. The activity is expressed as number of µmoles of α-naphthol or β-naphthol liberated per min.

3.4 Km and Vmax

Km and Vmax for esterase are determined by constructing a plot of 1/v against 1/[S].

3.5 pH Optima

The effect of pH on the activity of the purified esterase is studied using 50 mM buffers of different pH, (acetate buffer, pH 4.0, 4.5, 5.0 and 5.5, phosphate buffer, pH 6.0, 6.5, 7.0 and 7.5, Tris-HCl buffer, pH 8.0, 8.5 and 9.0 and carbonate buffer pH, 9.5, 10.0 and 11.0). The activity is measured using α-naphthyl acetate prepared in these buffers for 15 minutes.

3.6 pH Stability

The purified esterase is pre-incubated with 50 mM buffers of different pH (acetate buffer, pH 4.0, 4.5, 5.0 and 5.5, phosphate buffer, pH 6.0, 6.5, 7.0 and 7.5, Tris- HCl buffer, pH 8.0, 8.5 and 9.0 and carbonate buffer, pH 9.5, 10.0 and 11.0) for 30 mins at 4 ºC. Known aliquots from the incubated samples are removed and assayed using α-naphthyl acetate as a substrate for 15 mins at an optimum pH of 7.0.

3.7 Temperature Optima

The optimum temperature is determined by assaying the esterase activity at different temperatures ranging from 3, 7, 10, 19, 25, 30, 37, 42, 50, 60, 70 and 80º C using α-naphthyl acetate as substrate for 15 mins.

3.8 Temperature Stability

The purified esterase is pre-incubated at different temperatures ranging from 3, 7, 10, 19, 25, 30, 37, 42, 50, 60, 70 and 80ºC for 30 minutes, rapidly cooled to 0ºC and assayed using α-naphthyl acetate as substrate for 15 mins.

3.9 Inhibition Studies

Quantitative inhibition studies are carried out by preincubating the 0.5 ml of enzyme with 0.5 ml of different concentrations of inhibitors for 30 mins at 27º C before addition of 5 ml of substrate. Stock solutions of different inhibitors (2×10^{-2} M prepared in 50 mM phosphate buffer, pH 7.0, are serially diluted 10 times) are prepared to get the required concentrations (2×10^{-3} to 2×10^{-10} M). The residual esterase activity is measured. A plot of percent inhibition against pI is constructed. The effect of organophosphates (dichlorvos), carbamates (eserine sulfate) and p-chloromercuribenzoate (PCMB) on esterase activity using α-naphthyl acetate as substrate is determined, IC_{50} values for organophosphates (dichlorvos), p-chloromercuribenzoate (PCMB) and carbamates (eserine sulfate) are determined [27].

4. RESULTS AND DISCUSSION

The esterase enzyme is purified from the seeds of tamarind. The purified enzyme is shown to be homogenous by native PAGE (Fig. 1) and SDS-PAGE. The enzyme is characterized as carboxylesterase based on substrate specificity in conjunction with inhibitor specificity.

4.1 SDS – PAGE

The homogeneity of the esterase is confirmed by SDS-PAGE, which showed a single protein band, both in the presence and absence of β-mercaptoethanol, indicating that the purified esterase consists of a single polypeptide chain, corresponding to a molecular weight of 27.5 kD. This method is widely used for determination of the molecular weights of proteins. A straight line graph is obtained by plotting electrophoretic mobilities of standard proteins against log of their molecular weight (Fig.2).

4.2 Gel-Filtration on Sephadex G -100

The molecular weight of esterase by gel-filtration on sephadex G-100 is determined according to the method of Andrews (1970). A calibration curve is obtained by plotting Kav, [(Ve-Vo) / (Vt-Vo)], of standard proteins against their log molecular weight (Ve is elution volume, Vo is the void volume and Vt is the total volume of the gel). From this standard graph (Fig.3), the molecular weight of the purified esterase is calculated to be 26.9 kD.

4.3 Effect of Time on Esterase Activity

The rate of the reaction with time is determined at regular intervals of time for 30 mins. According to the plots of absorbancy against time (Fig.4), the reaction rate is linear during the entire course of the reaction of 30 mins.

4.4 Effect of Enzyme Concentration

The effect of different enzyme concentrations on the hydrolysis of α-naphthyl acetate by esterase is shown in Fig. 5. A linear relationship is observed from 2 µg to 10 µg protein used in the study.

4.5 Substrate Specificity

The activities of purified esterase towards different substrates are shown in Fig. 6. A, 7. A and Fig. 8. A. Among the substrates tested, the enzyme exhibited activity towards α- and β-naphthyl esters of acetate and α-naphthyl butyrate, but not towards α-naphthyl phosphate.

4.6 *Km* and *Vmax*

The enzyme is assayed at varying concentrations of α- and β-naphthyl acetate and α-naphthyl butyrate. From the Lineweaver-Burk plots (Fig. 6. B, 7. B and Fig. 8. B), *Km* and Vmax, are determined (Table 1).

4.7 Optimum pH & pH Stability

The effect of pH on the activity of purified esterase is studied using different buffers. The initial catalytic activity is determined with α-naphthyl acetate as the substrate. Suitable controls are prepared using heated enzyme to account for the non-enzymatic hydrolysis of the substrate. The catalytic activity of the purified esterase is markedly influenced by the pH and is maximum at pH 7 as shown in Fig.9. The purified esterase is found to be most stable between a pH 5 to 8 as shown in Fig.10.

4.8 Optimum Temperature, Temperature Stability and Energy of Activation

The reaction rates are determined at different temperatures ranging from 3º C to 80º C. The

plot of absorbency against temperature is shown in Fig.11. The esterase showed an optimum temperature of 40ºC. The esterase is found to be optimally stable from 5ºC upto 40ºC for 30 mins (Fig. 12). The straight line Arrhenius plot is obtained by plotting log v against 1/T (Fig.13). From the slope of the line, the energy of activation is calculated to be 7.16 kcal / mole.

4.9 Inhibitor Specificity

Organophosphates and carbamates are the two major classes of irreversible inhibitors of esterases. These inhibitors irreversibly bind to active site residue on these hydrolytic enzymes. The purified esterase is preincubated with different concentration of dichlorvos (organophosphate inhibitor), eserine sulphate (carbamate) and PCMB (p-chloromercuribenzoate, a sulphydryl inhibitor). A plot of percent inhibition against pI (negative log of molar concentration of inhibitor) gave sigmoid curves with respect to dichlorvos, but esterase is shown to be resistant towards esereine sulphate and PCMB (Fig. 14). The esterase is completely inhibited by dichlorvos at 10^{-3} M to 10^{-5} M and 90 % inhibited at 10^{-6} M and IC_{50} (inhibitor concentration showing 50 % inhibition) is 3.5 x10^{-6} M. However, the esterase is resistant to PCMB and eserine sulphate.

The purified carboxylesterase from the seeds of tamarind fall into the broad group of esterases that hydrolyse short-chain acyl compounds maximally. The marked inhibition by organophosphate (dichlorvos) and lack of inhibition by carbamate (eserine sulfate) and PCMB on the activity of esterase fulfill the criteria to categorize the esterase as carboxylesterases.

The esterase purified from the seeds of tamarind had a molecular weight of 27.5 kD as determined by SDS-PAGE, both in the presence and absence of β-mercaptothanol and is in close aggrement with the molecular weight determined by gel-filtration on Sephadex G -100 (26.9 kD). The results indicate the presence of a single polypeptide chain. Similarly, a single polypeptide chain is present in carboxylesterase purified from *Jatropha curcas* [28].

In general, the animal esterases are found to have high molecular weight, while the plant esterases have been reported to have lower molecular weight. The molecular weight of pig liver esterase has been reported to be 162-168 kDa and consisted of two subunits of types A and

B, which associates to form four trimers of the type AAA, AAB, ABB, and BBA [29]. Each subunit possessed a molecular weight of 55-60 kDa, and had different substrate specificities [30]. The bovine liver [31] and pig kidney [32] carboxylesterases are also reported to have two subunits possessing the same molecular weight as reported for pig liver esterase.

Unlike in the case of animal carboxylesterases, most plant carboxylesterases studied to date do not have the subunit structure. The carboxylesterases from finger millet [33] and sorghum [34] were reported to have a molecular weight of 60 kDa to 70 kDa and consisted of a single polypeptide chain. The carboxylesterase from the latex of *S. grantii* contained a single polypeptide chain and had a molecular weight 14 kDa [35]. Four carboxylesterases containing single polypeptide chains, with molecular weights in the range of 35 kDa to 50 kDa have been reported from the peel tissues of apple [36]. A unique carboxylesterase isozyme-z was isolated from the non-embryonic cell line of cultured carrot cells (*Dacus carota*), with a molecular weight of 35 kDa and was found to have a single polypeptide chain [37]. However, carboxyl esterases from cultured wheat cells [38] differ from the previously reported carboxylesterases in having two subunits with a subunit molecular weight of 22 kDa. Carboxylesterase isolated from the fruit of *Cucurbita maxima* also indicated two subunits with a subunit molecular weight of 18 kDa [39].

Animal and plant carboxylesterases studied so far fall into two distant categories – containing more than one subunit and a single subunit, respectively. The same is not true with insect carboxylesterases. Veerabhadrappa *et al.*, (1980) working with *H. cearulea* have reported a carboxylesterases of 102 kDa without any subunit structure [40]. Similarly, a carboxylesterase from intestine of the nematode *C. elegans* had a single polypeptide chain having a molecular weight of 60 kDa [41]. However, carboxylesterase from the silkworm consisted of two non-covalently associated polypeptide chains having a monomer molecular weight of 72 kDa as determined by SDS-PAGE and a molecular weight of 14.45 kDa as determined by gel-filtration on Sephadex G-200, respectively. Similarly, the esterase of *D. psuedoobscura* had a monomer molecular weight of around 80 kDa and it was hypothesized that this species can exist as dimer and tetramer, all the forms being active. Similarly, in the mosquito *Culex*

quenquefasciatus, carboxylesterase had a molecular weight of 62 kDa and is present in different polymeric forms. The enzyme is present in resistant strains of mosquito at a high concentration and in trace amount in suceptible strains and has been implicated in organophosphate detoxification [42]. In contrast, the carboxylesterase conferring OP resistance in aphid *Myzus persicae*, is a glycoprotein of 65 kDa [43].

The molecular weight described above indicated that they are typical oligomeric proteins with non-covalent association of subunits. However, a carboxylesterase isolated from the heamolymph of *Locusta migratoria* appear to be more complex and consisted of two subunits linked by disulphide bonds [44-45].

The carboxylesterases isolated from microorganisms are similar to insect carboxylesterases in so far as their subunit structure is concerned. For example, a carboxylesterase from bacteria *Yersinia pseudotuberculosis* is a pentamer. Similarly, the archaebacterium, *Sulpholobus acidocaldarius* contains a carboxylesterase which is a trimer with monomeric subunit of 32 kDa [46]. Conversely, carboxylesterase of *E. coli* has a single polylpeptide chain with a molecular weight of 57 kDa [47].

The purified esterase from the seeds of tamarind is proved to be carboxylesterase based on the substrate and inhibitor specificity. The esterase hydrolyzed both α- and β- naphthyl esters, but is inactive towards choline esters and naphthyl phosphate. Hence, the possibility of the esterase being cholinesterase and phosphatase is ruled out. This is further substantiated by complete

inhibition with organophosphate, but not carbamates. A plot of pI vs percent inhibition gave a single sigmoid curve confirming the presence of a single enzyme species. Since the esterase is resistant to sulphydryl reagent, but is sensitive to organophosphate, the esterase cannot be classified as an arylesterase. Hence, the purified esterase is classified as carboxylesterase.

The hydrolysis of naphthyl esters catalyzed by the carboxylesterase follows typical Michaelis Menten kinetics with no evidence of inhibition at high substrate concentrations. The carboxylesterase has a hydrophobic binding site or environment corresponding to the acyl group of the substrate (acetate and butyrate). This can be judged from the fact that there is very little change in *Km* values of naphthyl acetate and naphthyl butyrate. The affinity towards acetate and butyrate is almost same, irrespective of the length of the carbon chain of the acyl group. Same level of affinity (*Km*) is shown towards the leaving group of the substrate (α-and β-naphthol), and the binding site is big enough to accommodate either α- or β-naphthyl moiety. The lack of activity with compounds having polar leaving group, such as choline and phosphoryl group like phosphate indicate a very high hydrophobic environment at the active center of the of the enzyme.

The carboxylesterase is a typical enzyme possessing properties like pH optimum and temperature optimum similar to most of the carboxylesterases of animal, plant, insect and microbial systems. Sequencing the carboxylesterase and analysis by bioinformatics tool will provide an insight into the probable role of the tamaring seed carboxylesterase.
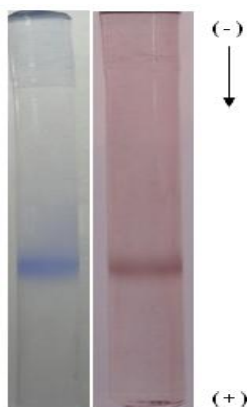

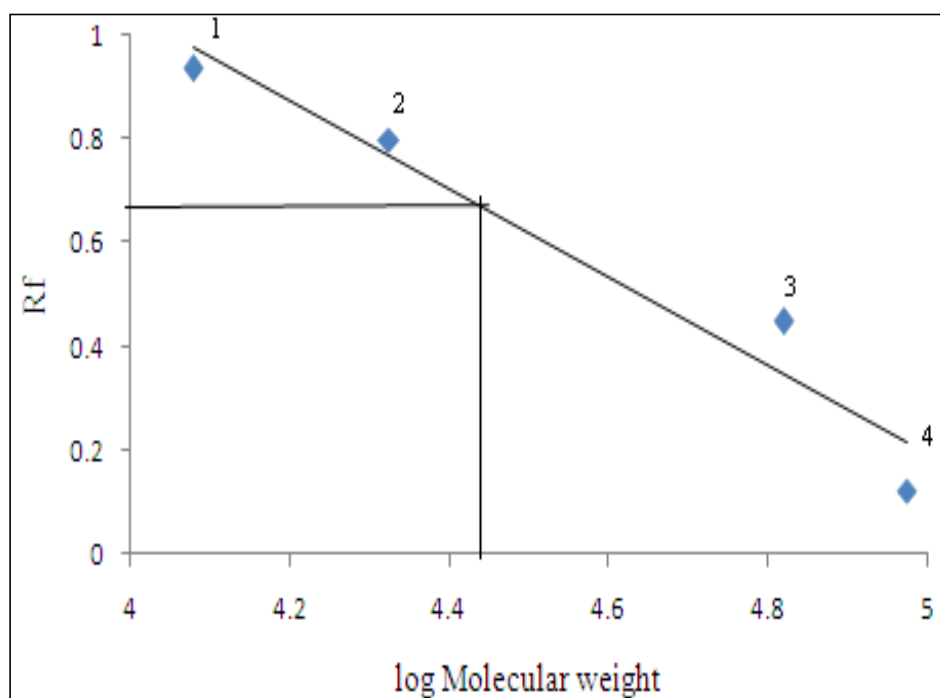**Fig. 1. Native PAGE pattern of purified esterase and protein of preparative PAGE fraction**

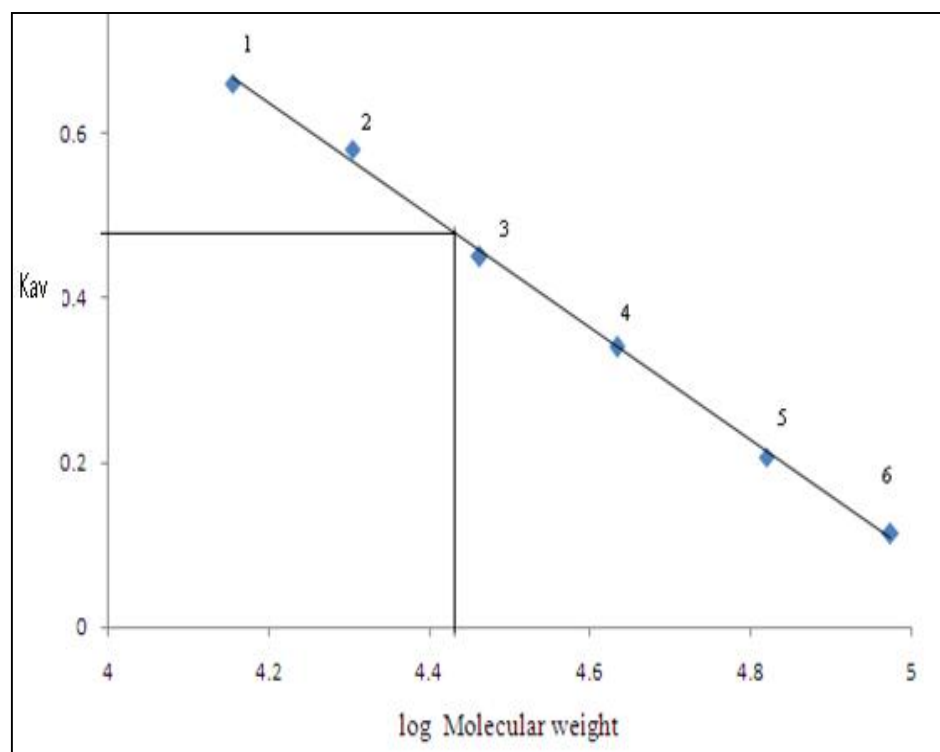**Fig. 2. Molecular Weight determination of purified esterse by SDS - PAGE**

Fig. 3. Molecular Weight determination of purified esterase by Gel filtration chromatography on sephadex G -100
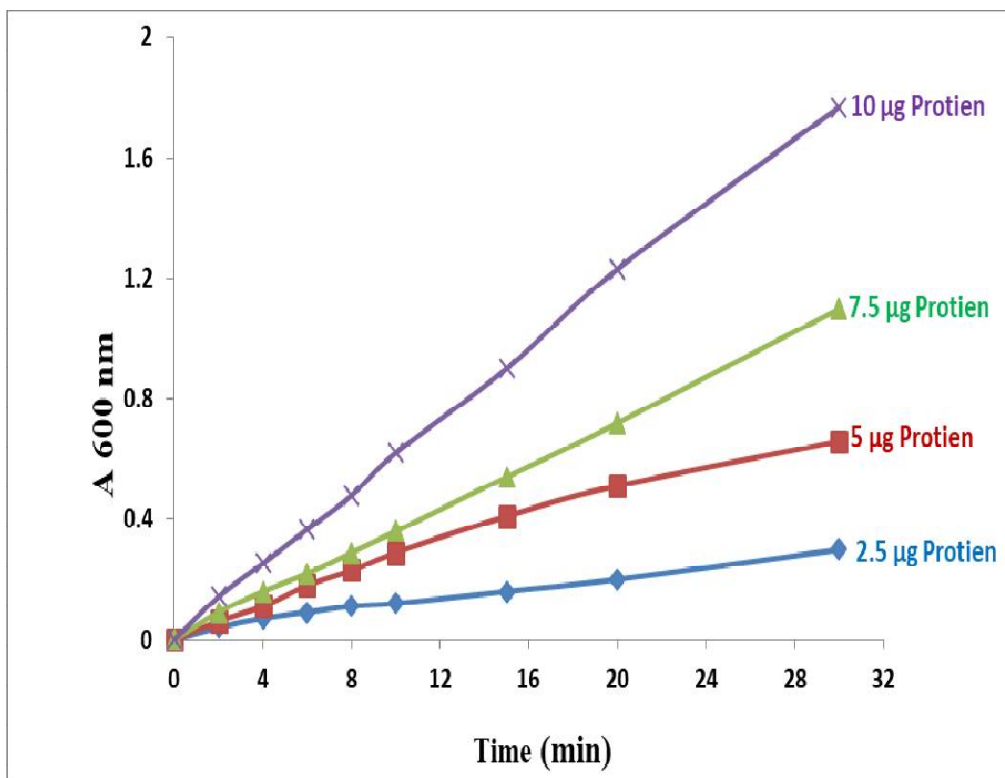
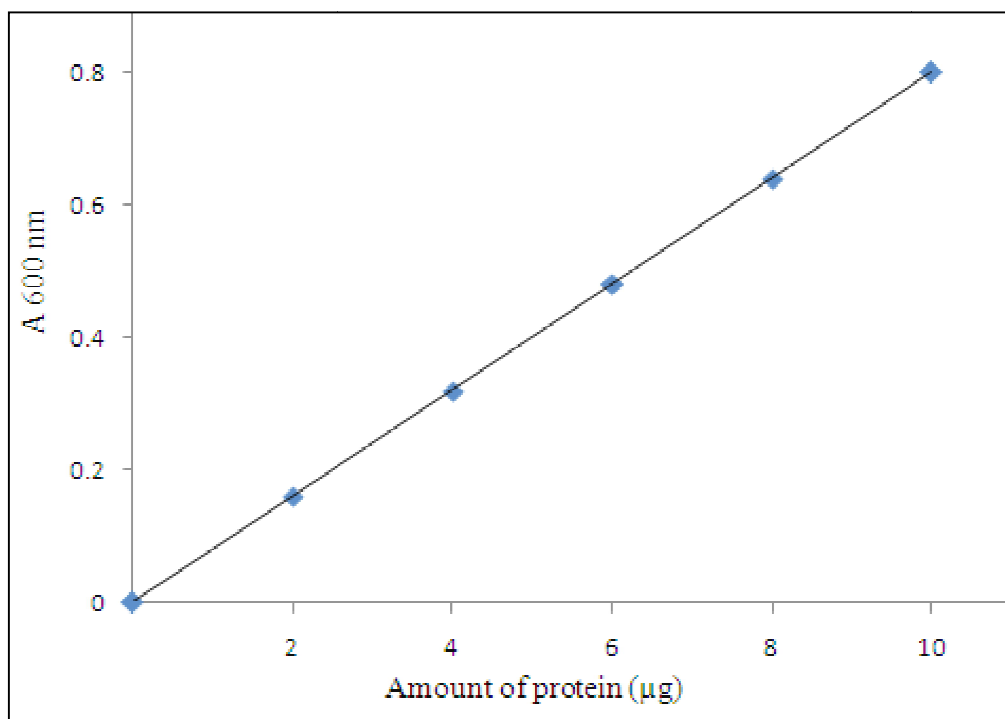**Fig. 4. Effect time on esterase activity at different protein concentration**

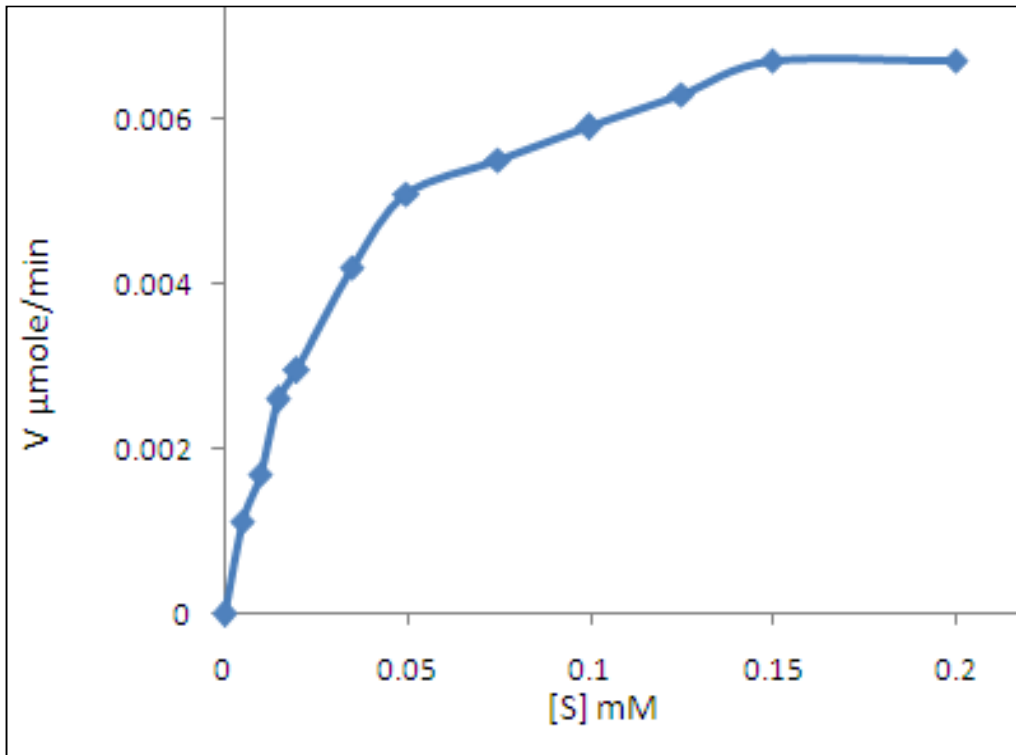**Fig. 5. Effect of protein concentration on esterase activity**

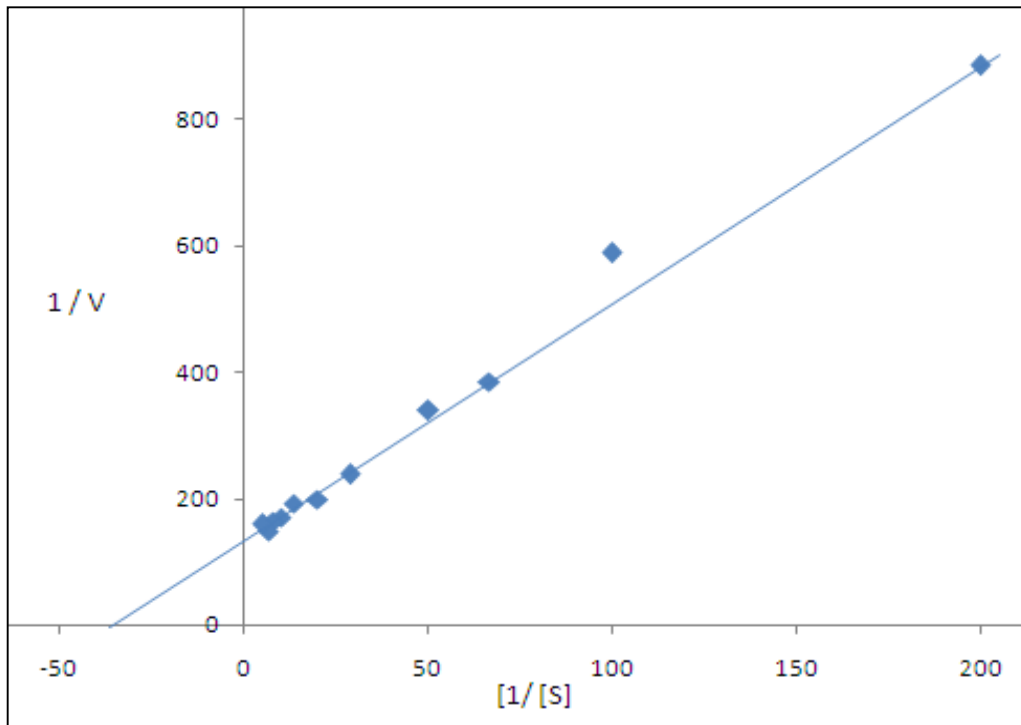**Fig. 6. A. Effect of α-naphthyl acetate concentration on esterase activity**
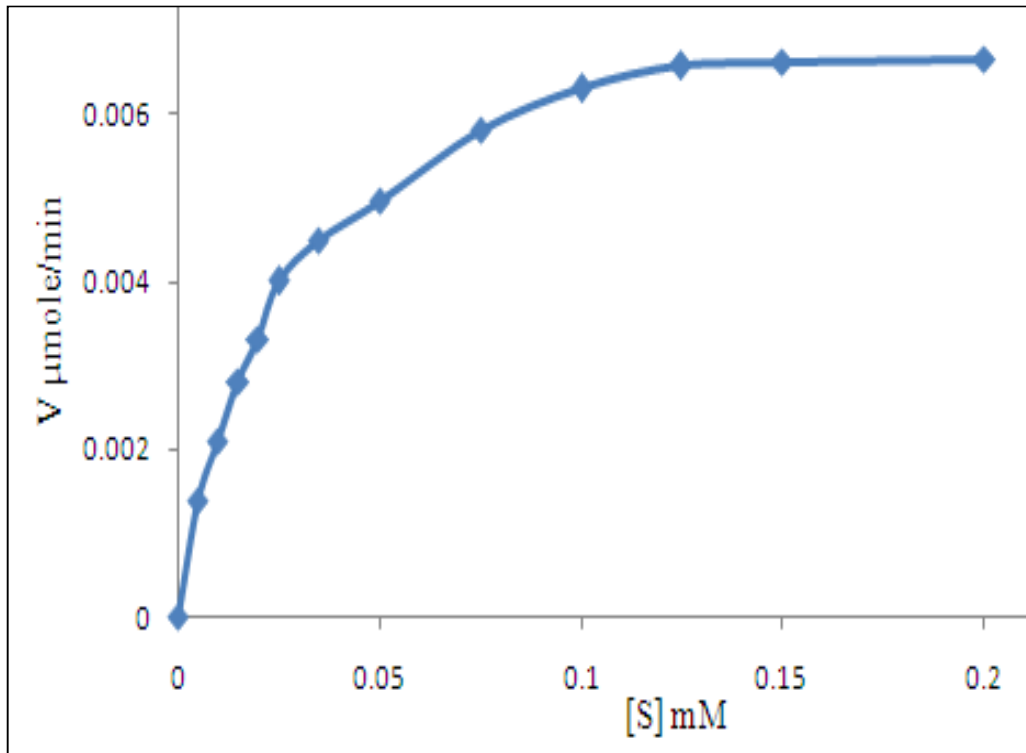

Fig. 6. B. *Km* & Vmax of esterase with α-naphthyl acetate

Fig. 7.A. Effect of β-naphthyl acetate concentration on esterase activity
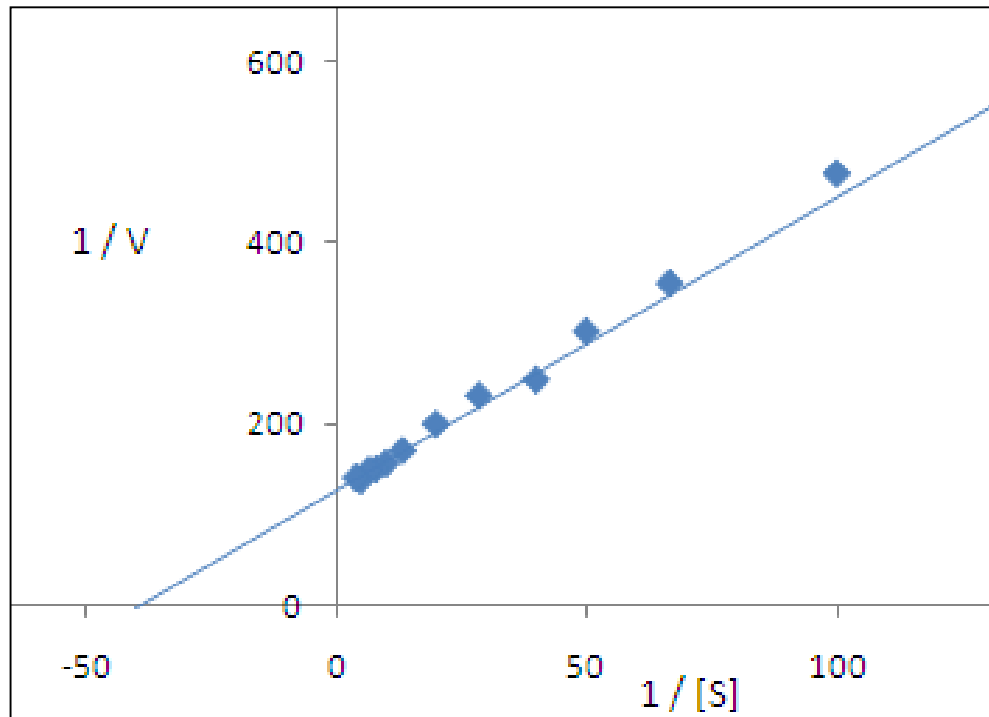
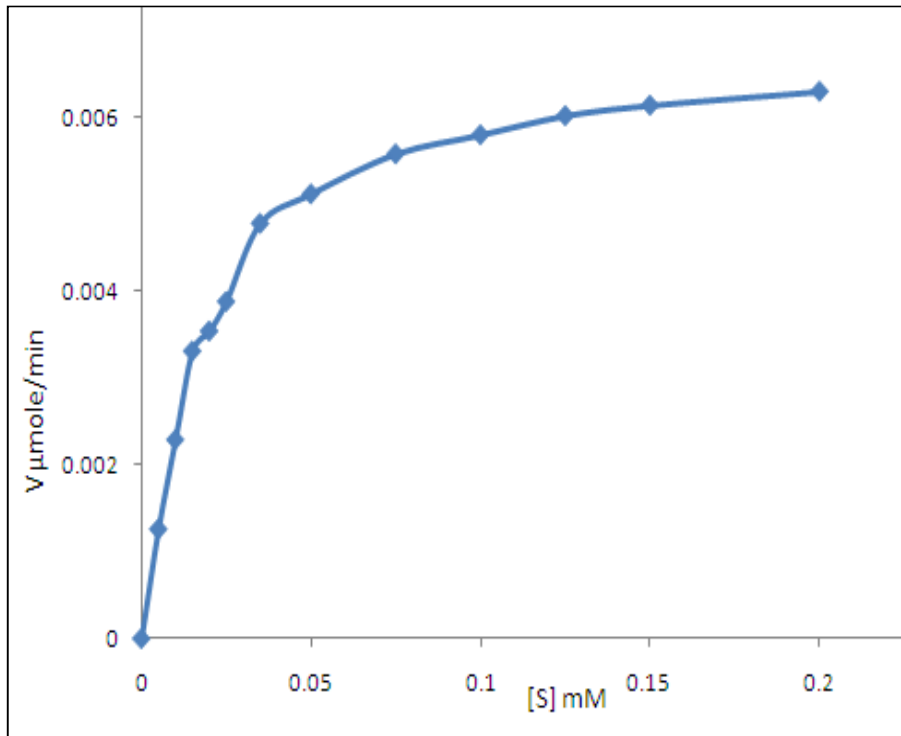**Fig. 7.B. *Km* & Vmax of esterase with β-naphthyl acetate**

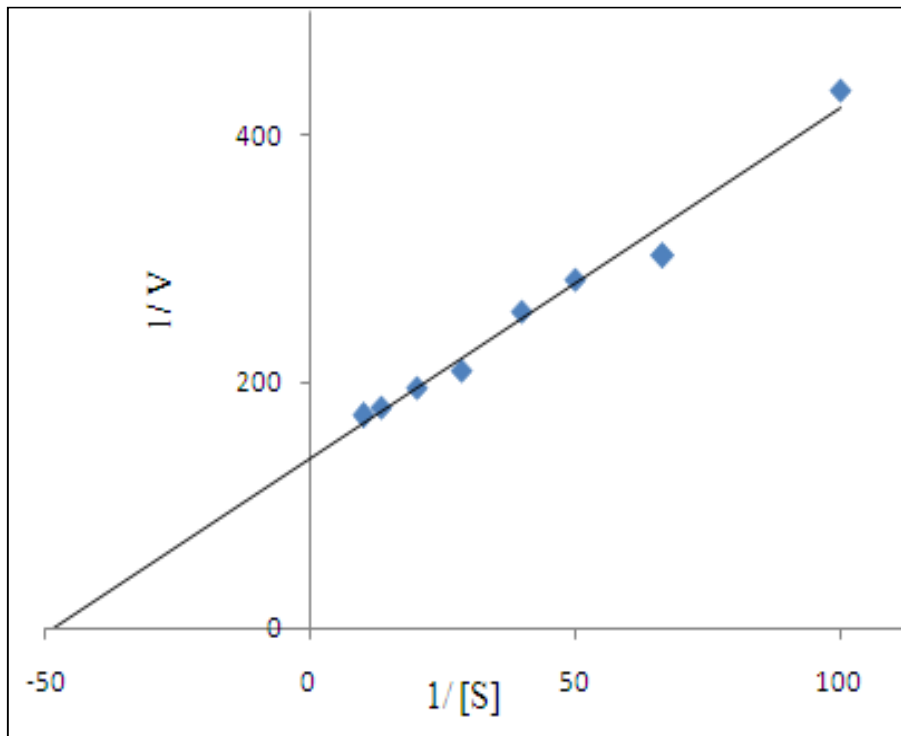**Fig. 8. A. Effect of α-naphthyl butyrate concentration on esterase activity**

Fig. 8. B. *Km* **& Vmax of esterase with α-naphthyl butyrate**

Table 1. *Km* and Vmax of purified esterase

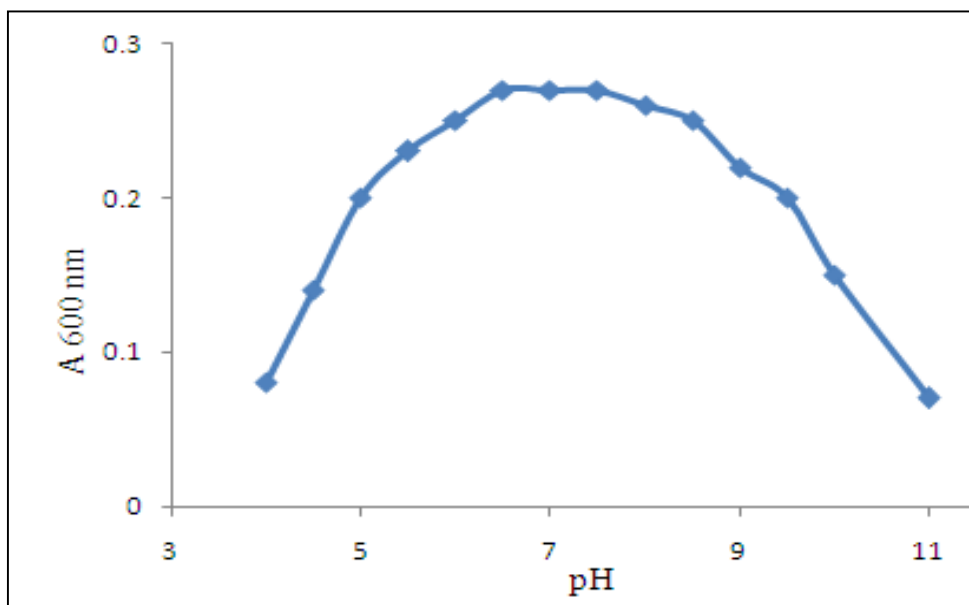| Substrate	Km (µM)	Vmax (µmole/min)
α– naphthyl acetate	28.6	7.1×10^{-3}
β- naphthyl acetate	22.2	7.41×10^{-3}
α- naphthyl butyrate	26.7	8.00×10^{-3}

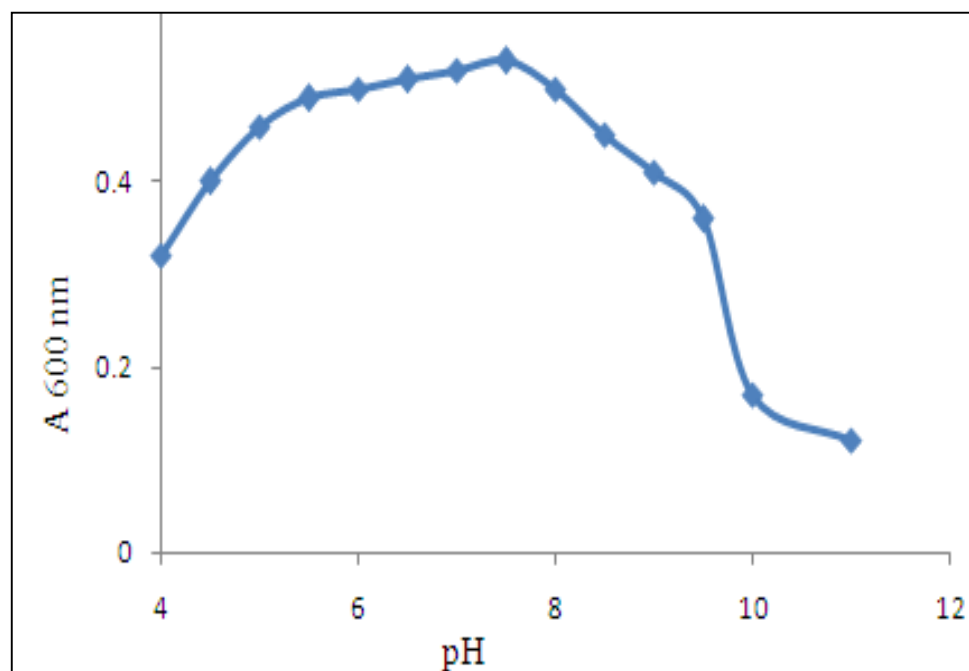**Fig. 9. Effect of pH on esterase activity**

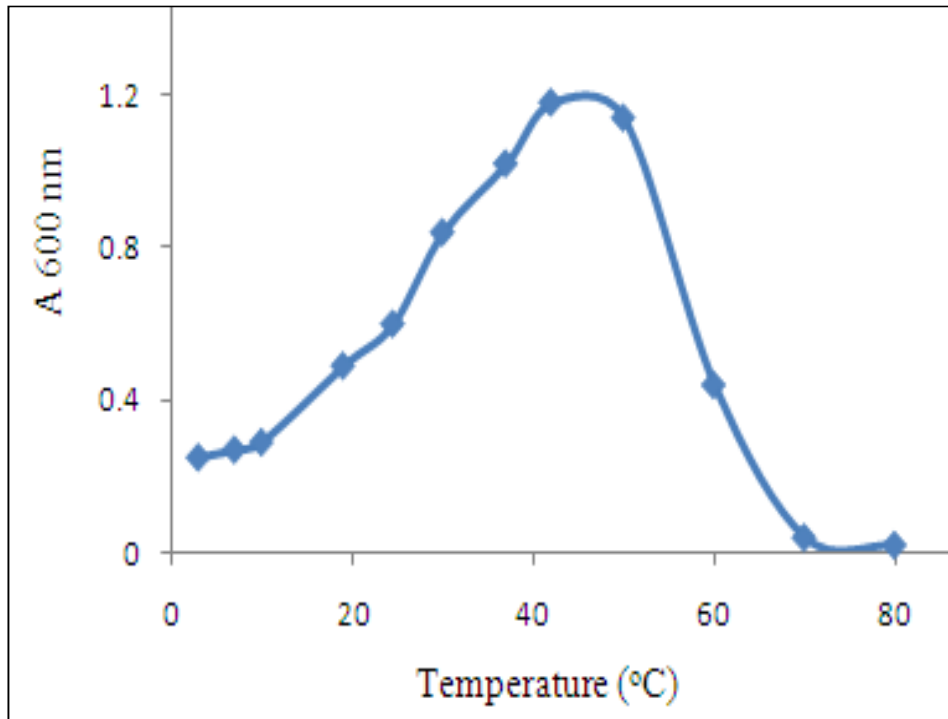

Fig. 10. pH stability of esterase

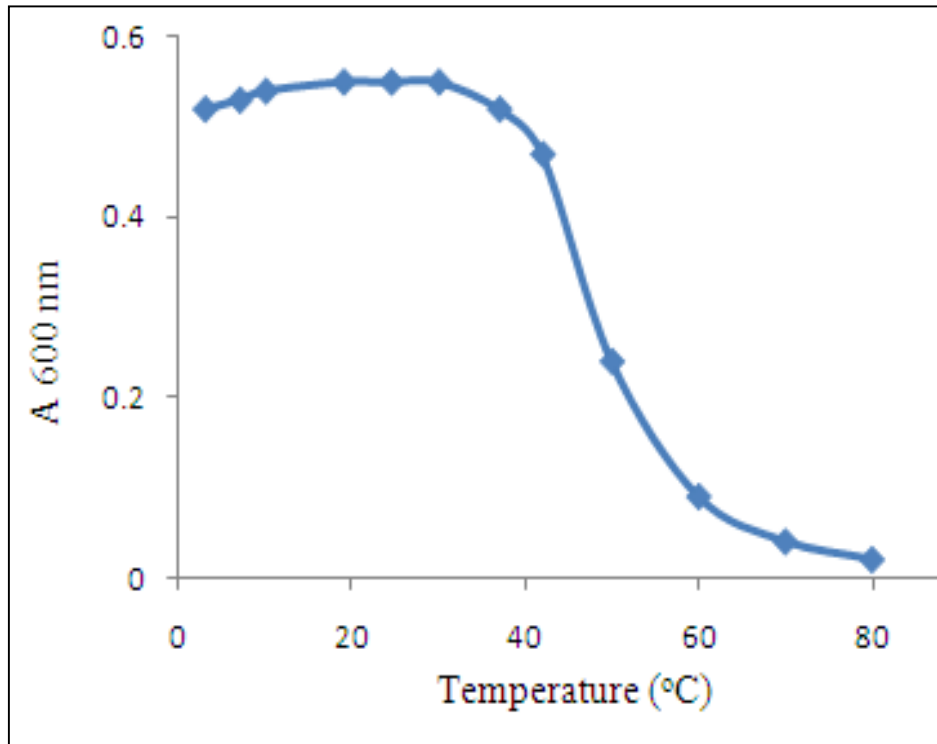**Fig. 11. Effect of temperature on esterse activity**
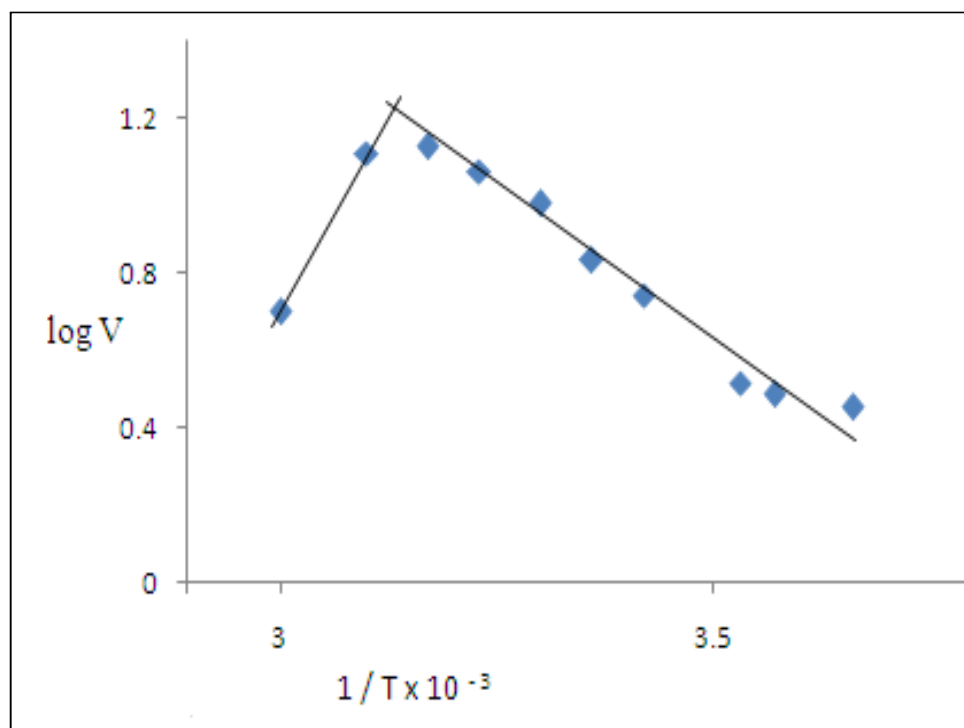

Fig. 12. Temperature stability of esterase
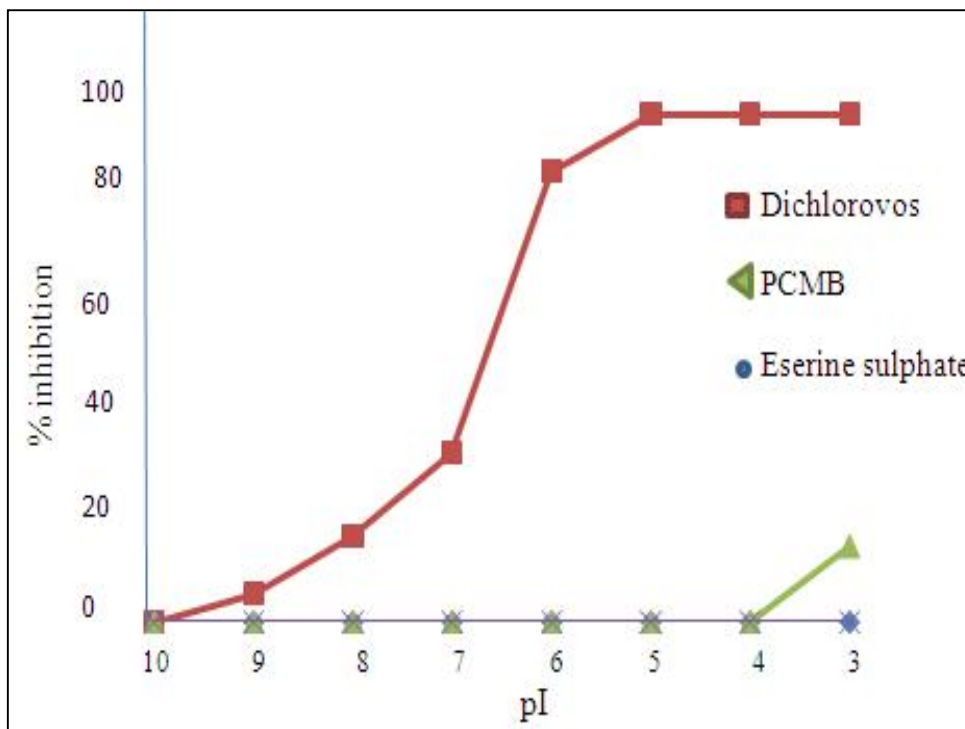
Fig. 13. Arrhenius plot for purified esterase

Fig. 14. Inhibition curve for the hydrolysis of α – naphthyl acetate by purified tamarind esterase

5. CONCLUSION

The carboxylesterases are a distinct group of enzyme molecules with overlapping substrate specificities. Based on the substrate and inhibitor specificity, the purified esterase from the seeds of *tamarindus Indica* was found to be carboxylesterase. The carboxylesterase is a typical enzyme possessing properties like pH optimum and temperature optimum etc., are similar to carboxylesterases found in all other organisms. Further work is essential to determine the physiological substrate and the role of this esterase in the growth and development of the plant.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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