Journal of Experimental Biology and Agricultural Sciences

http://www.jebas.org

ISSN No. 2320 – 8694

PURIFICATION AND CHARACTERIZATION OF ESTERASE FROM THE SEEDS OF *Caesalpinia mimosoides*

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Received – September 24, 2014; Revision – November 05, 2014, Accepted – December 26, 2014
Available Online – December 30, 2014

KEYWORDS

Non-specific esterases

Caesalpinia mimosoides seeds

Isolation

Partial purification

Physic-chemical properties

ABSTRACT

In the present study, esterase enzyme was isolated from the soaked seeds of *Caesalpinia mimosoides*. Partial purification was done employing conventional protein purification techniques such as salt fractionation and ion exchange chromatography on CM-cellulose. The specific activity and yield of the partially purified esterase was 1.25 and 09.01, respectively. SDS-PAGE was used to determine the molecular weight of the partially purified esterase and its molecular weight was found to be 20 kDa. The optimum pH and temperature of the partially purified esterase were 7.0 and 45^{o}C, respectively. The K_m and V_{max} for 1-naphthyl acetate are 0.11mM and 12.5 nmol/min, respectively.

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Peer review under responsibility of Journal of Experimental Biology and Agricultural Sciences.

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1 Introduction

Esterases (EC.3.1.1.1, carboxyl ester hydrolases) are hydrolytic enzymes; catalyze the hydrolysis of various types of esters. These are widely distributed in multiple forms, in plants, animals and microorganisms. In plants, these enzymes have a definite role, actively involved in growth and development, stomatal movement, insecticidal resistance against infection, fruit ripening, abscission, cell expansion, reproduction, secondary metabolite processes as well as hydrolysis of ester containing xenobiotic molecules (Dubey et al., 2000).

The natural substrates for the majority of esterases remain unknown, the activity being characterized using synthetic substrates, such as α- or β-naphthyl esters and p-nitrophenyl esters (Dubey et al., 2000). Esterases catalyze the hydrolysis of various types of exogenous and endogenous esters, preferably esters composed of short chain fatty acids (Bornscheuer, 2002). These enzymes exhibit a number of unique enzyme characteristics such as substrate specificity, region specificity and chiral specificity (Jung et al., 2003). The potential application of these enzymes for the synthesis of short chain esters has attracted the interest of a broad range of industrial fields like foods, pharmaceuticals and cosmetics. The significant functions of these esterases also include metabolism and subsequent detoxification of many agrochemicals, pharmaceuticals (Redinbo & Potter, 2005; Potter & Wadkins, 2006).

Caesalpinia mimosoides, a small spiny tropical trees or climbing shrubs belonging to family Fabaceae (subfamily:Caesalpinioideae), is mainly distributed in the South Asian countries like India, Myanmar, as well as in northern and north-eastern parts of Thailand. Young roots and leaves are edible and are traditionally used as a carminative and a remedy for dizziness (Napat Tangsaengvit et al., 2013).

In addition, the folk practitioners of Udupi district of India used the roots for treatment of ulcer and wound management, as well as for the treatment of arthritis. Furthermore, this plant showed in vivo anti-arthritic and analgesic activities. The methanolic extract of *C. mimosoides* shoot tips also exhibited antioxidant activity. Moreover, the aqueous and the ethanol extracts contained gallic acid, the anti oxidative compound.

Since *C. mimosoides* constitutes one of the potential sources of various phytochemicals and esterases might be involved in transesterification, detoxification and insecticide or pesticide scavenging activity, the present work was under taken to study the esterases to gain information regarding the biological role of these enzymes. In the present investigation, partial purification, characterization and properties of esterases isolated from the soaked seeds of *C. Mimosoides* were described.

2 Materials and Methods

2.1 Plant Material

The seeds of *Caesalpinia mimosoides* were collected from the Aranthoduand Kirlaya, SulliaTq, Dakshina Kannada , Karnataka.

2.2 Chemicals

Acetone, Acrylamide, N,N methylene bis acrylamide, CM-cellulose, ammonium per sulphate, 1-naphthyl acetate, Fast blue RR salt, FC Reagent, BSA, Coomassive Brilliant Blue R-250, Methanol, Acetic acid, ,β-mercaptoethanol were obtained from Sigma Chemical Company USA. All other chemical were of analytical grade. .

2.3 Crude Enzyme Extract

The seeds of *C. mimosoides* were soaked in distilled water for six hours and dehulled. The powder from the dehulled seeds (10%) was prepared according to the method given by Wetter (1957). The soaked dehulled seeds were blended with chilled acetone (10%), followed by filtration using suction pump. The cake obtained was dried at room temperature and powdered. It was stored at 0°C - 4°C until further use. A 10% crude enzyme extract of the acetone powder was prepared using 0.05 M sodium phosphate buffer pH 7.0 by stirring over a magnetic stirrer for 90 min at cold, followed by centrifugation at 10,000 rpm for 15 min at 4°C. The supernatant was collected for further analysis.

2.4 Purification

All the purification procedures were performed at 4^{o}C unless otherwise stated. To the crude enzyme extract, finely powdered ammonium sulphate was added to 0–30% saturation and the obtained precipitate was removed by centrifugation at 10,000 rpm for 30 min at 4^{o}C. For the supernatant obtained, finely powdered ammonium sulphate was added to get 30–80% saturation at 4^{o}C. The protein was precipitated by centrifugation at 10,000 rpm for 30 min. The precipitate thus obtained was dissolved in 0.05 M sodium acetate buffer, pH 5.3 and dialyzed against the same buffer.

The dialyzed sample was loaded onto a CM-cellulose column (1x30 cm) pre-equilibrated in 0.025 M sodium acetate buffer, pH 5.3. The fractions were collected at a flow rate of 30 mL/h with a fraction volume of 10 mL. The bound proteins were eluted by stepwise increase in ionic strength using start buffer containing 0.1 M, 0.3M and 0.5M NaCl. The quantitative and qualitative assay of protein and esterase for bound and unbound fractions were performed (Chandrashekharaiah et al., 2011).

2.5 Polyacrylamide Gel Electrophoresis (PAGE)

Anionic Native PAGE (Slab gel electrophoresis) was performed at pH 8.3 according to the method of Ornstein (1964). The electrophoresis was performed at 4°C for 3 h. After the run, the gels were removed and stained for esterase activity as described below (Chandrashekharaiah et al., 2011). The gels were stained for proteins using staining solution (0.02% coomassie brilliant blue R-250) and destained in methanol and acetic acid. SDS-PAGE (10% T) was performed after denaturing the proteins with SDS and β-mercaptoethanol. The gels were stained and destined as before.

2.6 Enzyme Assay

Esterase activity was assayed according to the method of Gomori (1953) as modified by Van Asperen (1962). The assay mixture consisting of 5 mL of 0.3 mM 1-naphthyl acetate (a stock solution of 30 mM 1-naphthyl acetate prepared in acetone and diluted in 0.05 M sodium phosphate buffer pH 7.0) and 50–100 µg of enzyme was incubated at 27 ° C for 15 min. The reaction was stopped by the addition of 1 mL of DBLS reagent (2 parts of 2% diazo blue B and 5parts5%sodium lauryl sulphate). In the control, enzyme was inactivated by DBLS prior to incubation with substrate. The absorbance of the developed colour was measured at 600 nm. One unit of enzyme activity was defined as the amount of enzyme that released 1 nmol of product per min at pH 7.0 at 27°C. Protein concentration was determined according to the method of Lowry et al. (1951), using bovine serum albumin(BSA) as standard. The protein content in the fractions obtained from chromatographic columns was routinely monitored by measuring absorbance at 280 nm.

2.7 Staining of Esterase Activity

Esterase activity on polyacrylamide gels was detected by the method of Hunter & Markert (1957). The gels were stained for esterase activity after electrophoresis in a solution containing 50 mL of 0.05 M sodium phosphate buffer , pH 7.0, 50mg of Fast blue RR and 20 mg of 1-naphthyl acetate (dissolved in 1 mL of acetone) for 15 min at 27℃. The gels were stored in 7% acetic acid solution.

2.8 Molecular Weight Determination

The molecular weight of the native enzyme was determined according to the method of Andrew (1970) using Sephadex - G-150 (0.9 9 115 cm) pre-equilibrated with 0.05 M sodium phosphate buffer pH 7.0, at a flow rate of 12 mL/h. The column was calibrated using cytochrome-c (12.2 kDa), carbonic anhydrase (29 kDa), BSA (66 kDa), alcohol dehydrogenase (150 kDa) and β-amylase (200 kDa). Blue Dextran (2,000 kDa) was used to determine the void volume (Vo). The molecular weight of the esterase was determined from the plot of log molecular weight versus Rf value. The molecular weight of the partially purified esterase was also determined by SDS-PAGE from the graph of molecular weight against relative mobility.

2.9 Kinetic Studies

2.9.1 Effect of Time

The partially purified esterase was incubated with 1-naphthyl acetate for 0,1,3,5, 8, 10,12,15,20 minutes at 27 ^{0}C. The esterase assay was performed as mentioned above. The amount of 1-naphthol released for each time of incubation was determined from the standard graph of1-naphthol.

2.9.2 Enzyme Concentration

A typical esterase assay as described previously was carried out for partially purified esterase containing 0, 20, 40, 60, 80 and100 µg of proteins. The amount of 1-naphthol released in each case was determined as described previously.

2.9.3 Effect of pH and Temperature

The Optimum pH of the partially purified esterase was studied using sodium acetate buffer (pH 4.0), Sodium citrate buffer (pH 5.0), Sodium phosphate buffer (pH 6, 7 and 7.5), tris-Hcl buffer (pH 8.0 & 9.0). The catalytic activity of the enzyme was determined in each case using 1-naphthyl acetate as substrate.

The pH stability of the partially purified esterase was determined by incubating with buffer of different pH (0.2 M, pH4.0–9.0) at 27°C. After incubation, the activity was determined as described above and 1-naphthol formed was determined calorimetrically. Similarly, the Optimum temperature on the activity of the partially purified esterase was studied at different temperatures ranging between 4°C and 60^{0}C. The enzyme was diluted suitably and incubated with substrate, 1-naphthyl acetate at different temperature for 15 min. The amount of 1-naphthol released was measured calorimetrically.

The temperature stability of the partially purified esterase was studied by incubating the enzyme at different temperature (4°C – 60°C) for 15 min. The incubated samples were rapidly cooled and assayed at optimum temperature. The amount of 1-naphthol released was measured calorimetrically.

2.9.4 K_m and V_{max}

The purified esterase was incubated with different concentrations of 1-naphthyl acetate for 15 min at optimum temperature and the assay was carried out as described previously. The 1-naphthol formed in each concentration was determined calorimetrically.

Table 1 Purification of esterase from soaked seeds of *Caeselpania mimosoides*

Purification Steps	Total protein (mg)	Total Activity (nmol/Min)	Specific Activity(IU/mg)	Folding Purification	% yeild
Crude	726	90	0.123	1	100
Ammonium Sulphate Fractionation 30-80%	536.9	172	0.3208	2.601	73.953
CM-Cellulose Ion Exchange Chromatography	65.430	729.03	1.14	9.268	9.01

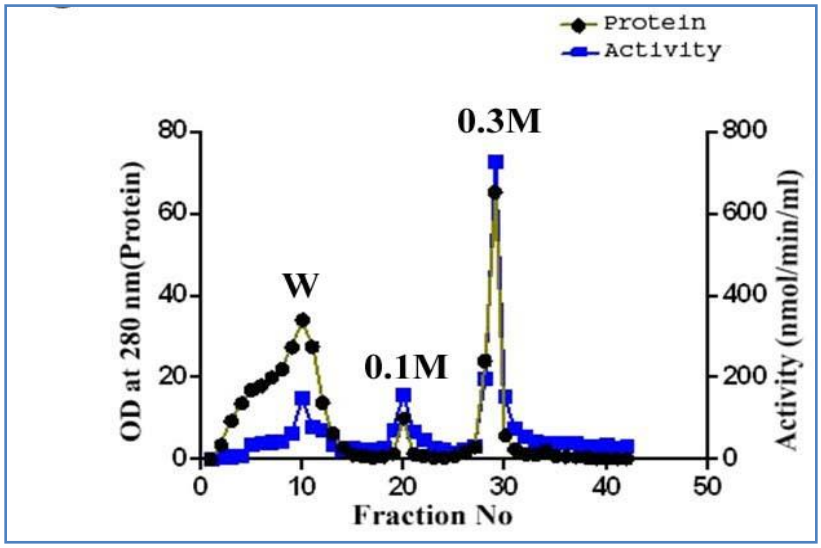

Figure.1 Elution profile of esterases from the soaked seeds of C.mimosoides on CM cellulose using 0.025 M sodium acetate buffer pH 5.3. The proteins were eluted by stepwise increase in the ionic strength using 0.1 M and 0.3 M sodium chloride.

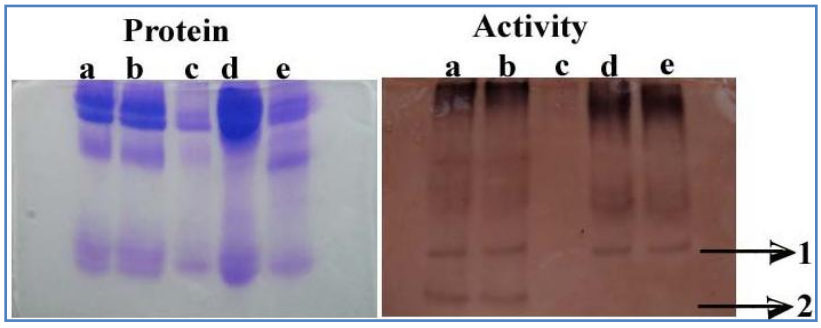

Figure 2 (2a & 2b) Native PAGE pattern of C.Mimosoides seed protein and Esterase; (a&b) crude, (c) 0-30%, (d) 30-60%, and (e) 60-80% Ammonium sulphate fractionation.

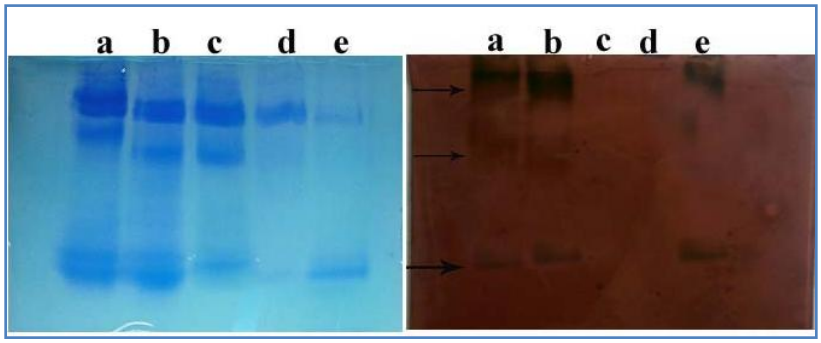

Figure 3 (a &b) Native PAGE pattern of Partialy purified C.Mimosoides seed protein and Esterase; (a) crude,(b) Ammonium sulphate,(c) washing,(d) 0.1 Fraction, (e) 0.3 Fraction.

3 Results and Discussion

Non-specific esterases represent a large, diverse and complex group of major hydrolytic enzymes and posses the property of overlapping substrate specificity, hydrolysing both endogenous and exogenous esters of widely differing structures. Esterases were isolated and purified from various sources including plants, animals, and microrganisms. Multiple forms of esterase catalyzing the hydrolysis of carboxylic esters of short chain fatty acids have been demonstrated in several plant tissues including seeds (Schwartz et al., 1964) and leaves of *Phaseolus* species (Rudolph & Stahmann, 1966). The crude enzyme extract from the seeds of *C. mimosoidaes* was subjected to ammonium sulphate fractionation (0 to 30% and 30 to 80%). The ammonium sulphate fraction was subjected to CM-cellulose chromatography. The elution profile of CM-cellulose chromatography is shown in Figure. 1.

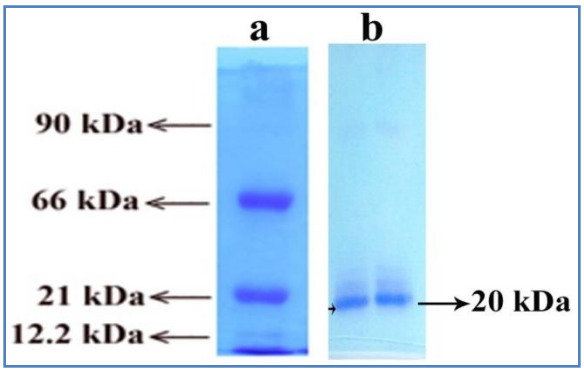

Figure 4 Molecular Weight Determination.SDS–PAGE pattern of (a) standard proteins (b) partially purified Caeselpinia mimosoide sseed esterase in the presence and (c) absence of β-mercaptoethanol.

Two peaks of esterase activities were eluted and were designated as fraction I and fraction II (fraction I was minor and fraction II was major peak). Fraction I was eluted by 0.1 M sodium chloride and fraction II by 0.3 M sodium chloride in starting buffer. Results of the purification showing the recovery, fold purification and the specific activity at each purification stage is given in Table 1.

The CM-cellulose fraction II after staining for esterases showed two esterolytic bands (one was major and other was minor). The esterases were partially purified to about 9.2 fold with 9% recovery. The esterases have been purified from various plant sources including *Cucurbita maxima* fruit tissue (Nourse et al., 1989), *Avena fatua* (Mohamed et al., 2000), finger millet (*Eleusine coracana*) (Liu et al., 2001), tomato (Stuhlfelder et al., 2002) and *Cucurbita pepo* (Fahmy et al., 2008) by employing different purification processes including ammonium sulphate fractionation, ion exchange chromatography and gel filtration chromatography. Two carboxylesterases from the seeds of *Mucuna pruriens* were purified to apparent homogeneity employing ammonium sulphate fractionation, CM-cellulose chromatography, gel-permeation chromatography (Chandrashekharaiah et al., 2011).

The velocity of the enzyme catalyzed reaction with time was analyzed and the activity was linear up to 20 min for *Caesalpinia* seed esterase (Figure.5a). The effect of enzyme concentration on the hydrolysis of 1-naphthyl acetate by partially purified esterase of *C. mimosoides* was analyzed and the linearity was obtained up to 100 µg of protein. The enzyme hydrolyzed 1-naphthyl acetate exhibited specific activities of 0.32 nmol/min/ mg protein.

Most of the purified plant esterases have low molecular weights and contain single polypeptide chain. The molecular weight of esterase of *C. Mimosoides* seeds as determined by SDS-PAGE was found to be 20 kDa (Figure. 4), both in presence and absence of 2-mercaptoethanol. The results of molecular weight determination obtained by SDS-PAGE indicate the monomeric nature of the isolated esterase. A low molecular weight esterases, each containing a single polypeptide chain were purified and characterized from various plant sources including apple, *Synadrium grantii* and *M. Pruriens* (Bartley & Stevens, 1981; Govindappa et al., 1987; Chandrashekharaiah et al., 2011). Most of the carboxylesterases studied so far, have low pI values contain large proportions of acidic amino acids (Murthy & Veerabhadrappa, 1996). The binding affinity to cation exchanger probably indicates the presence of large proportions of basic amino acids and hence the basic isoelectric pH to this enzyme. Chandrashekharaih et al., (2011) reported two esterases binding affinity to cation exchanger from the seeds of *M. pruriens*.

The hydrolysis of naphthyl ester such as 1-naphthyl acetate catalyzed by *C. mimosoides* esterase follows typical Michaelis-Menten kinetics with no evidence of inhibition at high substrate concentrations. The Km and Vmax values were determined from the Line weaver-Burk plot (Figure. 5d). The Km and Vmax for 1-naphthyl acetate are 0.11mM and 12.5 nmol/min, respectively. The physiological substrate of *C. mimosoides* esterase is not known and only naphthyl ester was used in the present investigation. Substrate specificity studies of purified fractions of various plant sources including finger millet, bean, pea, *Synandrium grantii* and Mucuna seed esterases with phenyl esters, glyceryl esters and naphthyl esters (Montgomery et al., 1968; Veerabhadrappa & Montgomery, 1971; Govindappa et al., 1987; Chandrashekharaiah et al., 2011) revealed that in all these cases, esterases hydrolyzed short chain esters exhibiting preferential action toward propionyl esters.

On the other hand, the rate of ester hydrolysis catalyzed by the partially purified apple esterases increased with increase in the carbon number of the substrate in the order C2–C6, but activity declined with higher molecular weight esters.

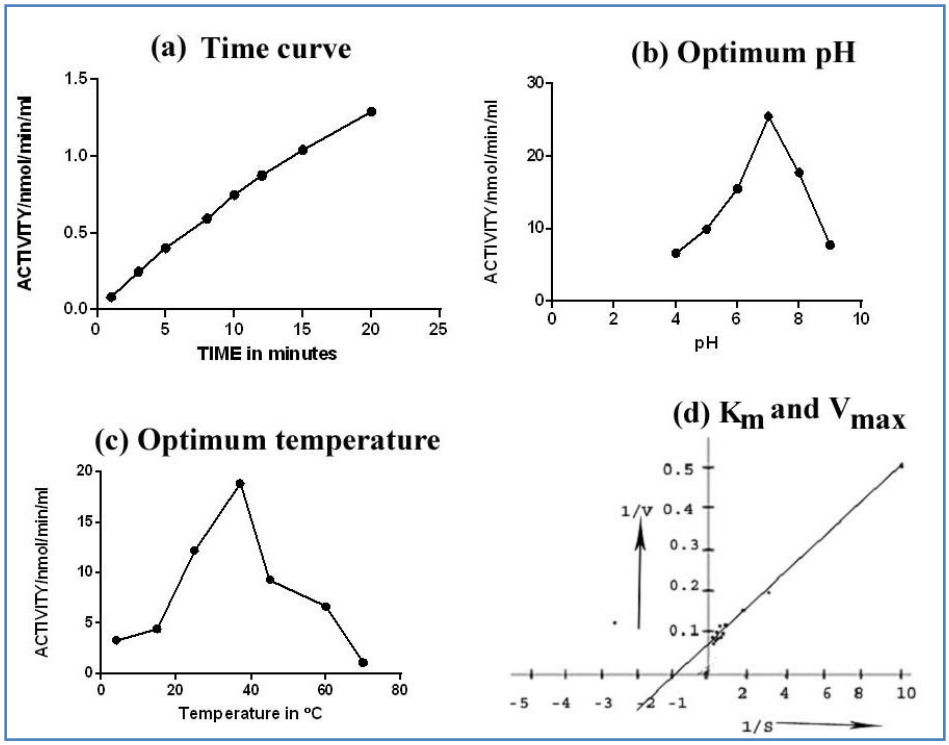

Figure 5 (a) Effect of time on the activity of partially purified Caeselpinia seed esterase; (b) Effect of pH of partially purified Caeselpinia seed esterase; (c) Effect of temperature partially purified Caeselpinia seed Esterase; (d) Determination of K_m and V_{max} of partially purified Caeselpinia seed Esterase

This activity was interpreted to be responsible for the hydrolysis of carboxylic acid esters during the ripening of apples (Bartley & Stevens, 1981). Esterases with more affinity towards the short chain naphthyl esters were reported from finger millet and Mucuna seeds Upadhya et al., 1985; Chandrashekharaiah et al., 2011). This activity was interpreted to be responsible for the hydrolysis of short chain fatty acid esters during growth and development of the plant. In the present investigation, *Caesalpania* seed esterase activity may be important for the hydrolysis of short chain fatty acid esters during growth and development.

The optimum pH obtained for most of the purified esterases of plants and animals ranged from pH 7.0 to 9.0. Among plant esterases, an optimum pH of 7.0 was obtained for sorghum, barley and Mucuna seeds (Burger et al., 1970; Sae et al., 1971; Chandrashekharaiah et al., 2011) and 7.5 for finger millet, *Synadenium grantii* and Mucuna seeds (Upadhya et al., 1985; Govindappa et al., 1987; Chandrashekharaiah et al., 2011). Most of plant esterases were stable between pH 4 and 9.0. Similarly, an optimum pH of 7 (Figure. 5b) was obtained for *Caesalpania* seed esterase and the enzyme was stable between pH 4 and 9.0. The purified *Caesalpinia* seed esterase was found to be optimally active at 45 ◦C (Figure.c) and stable up to 60°C .The optimum temperature obtained for other plant esterases such as barley, finger millet, *Synadenium grantii, C. pepo,* and *M. pruriens,* was also in the above range (Burger et al., 1970; Upadhya et al., 1985; Govindappa et al., 1987; Fahmy et al., 2008; Chandrashekharaiah et al., 2011).

Conclusion

The esterases are large diverse and complex group of enzyme molecules with overlapping substrate specificities and very little is known about their natural substrates. However, all esterases that have been studied in detail exhibited maximal activity towards short chain esters containing a particular acyl group. The Caesalpinia seed esterase, falls into a broad class of esterases that hydrolyze maximally shorter chain acyl esters. The Km and Vmax of Caesalpinia seed esterase for 1-naphthyl acetate indicated that, these enzymes exhibits higher activity towards short chain naphthyl esters. Therefore, Caesalpinia seed esterase may have a considerable role in the hydrolysis of short chain fatty acid esters during growth and development of the plant.

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