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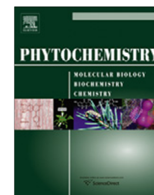

Carboxylesterases from the seeds of an underutilized legume, *Mucuna pruriens*; isolation, purification and characterization

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ABSTRACT

Two carboxylesterases (ME-III and ME-IV) have been purified to apparent homogeneity from the seeds of *Mucuna pruriens* employing ammonium sulfate fractionation, cation exchange chromatography on CM-cellulose, gel-permeation chromatography on Sephadex G-100 and preparative PAGE. The homogeneity of the purified preparations was confirmed by polyacrylamide gel electrophoresis (PAGE), gel-electrofocussing and SDS–PAGE. The molecular weights determined by gel-permeation chromatography on Sephadex G-200 were 20.89 kDa (ME-III) and 31.62 kDa (ME-IV). The molecular weights determined by SDS–PAGE both in the presence and absence of 2-mercaptoethanol were 21 kDa (ME-III) and 30.2 kDa (ME-IV) respectively, suggesting a monomeric structure for both the enzymes. The enzymes were found to have Stokes radius of 2.4 nm (ME-III) and 2.7 nm (ME-IV). The isoelectric pH values of the enzymes, ME-III and ME-IV, were 6.8 and 7.4, respectively. ME-III and ME-IV were classified as carboxylesterases employing PAGE in conjunction with substrate and inhibitor specificity. The K_m of ME-III and ME-IV with 1-naphthyl acetate as substrate was 0.1 and 0.166 mM while with 1-naphthyl propionate as substrate the K_m was 0.052 and 0.0454 mM, respectively. As the carbon chain length of the acyl group increased, the affinity of the substrate to the enzyme increased indicating hydrophobic nature of the acyl group binding site. The enzymes exhibited an optimum temperature of 45 °C (ME-III) and 37 °C (ME-IV), an optimum pH of 7.0 (ME-III) and 7.5 (ME-IV) and both the enzymes (ME-III and ME-IV) were stable up to 120 min at 35 °C. Both the enzymes were inhibited by organophosphates (dichlorvos and phosphamidon), but resistant towards carbamates (carbaryl and eserine sulfate) and sulphydryl inhibitors (p-chloromercuricbenzoate, PCMB).

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1. Introduction

Carboxylesterases (EC.3.1.1.1, carboxyl ester hydrolases) are enzymes belong to the group of hydrolases catalyze the hydrolysis of various types of both endogenous and exogenous esters. These are widely distributed in nature, found in animals, plants and microorganisms. They occur in multiple molecular forms and exhibit a number of unique enzyme characteristics such as substrate specificity, regiospecificity and chiral specificity (Jung et al., 2003). The functions of these enzymes have also been implicated in carbon source utilization, pathogenicity and detoxification (Ewis et al., 2004). These enzymes preferably catalyze the hydrolysis of esters composed of short chain fatty acids, but they also can catalyze ester synthesis and transesterifications (Bornscheuer, 2002). Particularly, 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. Among these flavor acetates from primary alcohols constitute compounds with a great application due to their characteristic fragrance and flavor (Romero et al., 2005). The carboxylesterases are also involved in fruit ripening, abscission, cell expansion, reproduction as well as hydrolysis of ester containing xenobiotic molecules.

Other significant functions of the carboxylesterases include metabolism and subsequent detoxification of many agrochemicals, pharmaceuticals (Redinbo and Potter, 2005; Potter and Wadkins, 2006), metabolism of a number of therapeutics (Williams, 1985), including the cholesterol-lowering drug, lovastatin (Tang and Kalow, 1995), the antiinfluenza drug, Oseltamivir (Tamiflu) (Shi et al., 2006), the narcotic analgesic meperidine (Demerol) (Zhang et al., 1999), cocaine and heroin (Pindel et al., 1997), and resolution of racemic mixtures by transesterification, or the enantioselective hydrolysis of esters for obtaining optically pure compounds (Bornscheuer, 2002). Carboxylesterase activity is also used extensively in soft- and pro-drug design (Bodor and Buchwald, 2000, 2003, 2004). However, the natural substrates for the majority of carboxylesterases remain unknown, the activity being characterized using synthetic substrates, such as α- or β-naphthyl esters and p-nitrophenyl esters (Dubey et al., 2000). In addition to carb-

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oxylester hydrolase activity, reports have shown that some carboxylesterase enzymes possess amidase, dehydratase and phosphatase activity as they can utilize acetanilide, hydroxyisoflavones and organophosphates respectively (Leinweber, 1987; Oakeshott et al., 1999).

The genus *Mucuna* belongs to the family Fabaceae (Leguminoceae) which contains up to 150 species of annual and perennial legumes of pan tropical distribution. Mucuna is extensively used as cover crop to control insects and weeds in agriculture. Mucuna pods are covered with reddish-orange hairs, which readily dislodge and cause intense skin irritation and itch due to presence of a chemical called Mucunain. Many varieties and accessions of the wild legume, *Mucuna* are in great demand in food and pharmaceutical industries. The nutritional importance of *Mucuna* seeds as a rich source of protein supplement in food and feed has been well documented (Siddhuraju et al., 2000; Siddhuraju and Becker, 2001; Bressani, 2002).

All parts of *Mucuna* plant are known to possess high medicinal value (Caius, 1989; Warrier et al., 1996). *Mucuna pruriens* has been reported to contain several useful phytochemicals (Morris, 1999). Alkaloid screening resulted in confirmation of the presence of 5-methoxytryptamine in all the samples tested and serotonin confined to fresh leaves and stems (Szabo, 2003). Various compounds present in pods, seeds, leaves and roots of *Mucuna* include bufotenine, choline, *N*, *N*-dimethyltryptomine, 5-oxyindole-3-alkylamines, indole-3-alkylamine and B-carboline (Ghosal et al., 1971). Since *Mucuna* 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 undertaken to study the carboxylesterases to gain information regarding the biological role of these enzymes. In the present investigation, purification, characterization and properties of two carboxylesterases isolated from the soaked seeds of *M. pruriens* are described.

2. Results and discussion

2.1. Purification

Carboxylesterases were purified and characterized from different sources including plants, animals, and microrganisms. They have been purified from various plant sources including finger millet (*Eleusine coracana*) (Upadhya et al., 1985), *Cucurbita maxima* fruit tissue (Nourse et al., 1989), *Jatropa curcas* L. seeds (Staubmann et al., 1999), *Avena fatua* (Mohamed et al., 2000), tomato (Stuhlfelder et al., 2002) and *Cucurbita pepo* (Afaf S Fahmy et al., 2008) by employing different purification processes including ammonium sulphate fractionation, ion exchange chromatography and gel filtration chromatography. However, not all preparations have been shown to be homogeneous. Govindappa et al. (1987) purified one of the carboxylesterases from the latex of *Synadenium grantii* by acetone fractionation, CM – Sephadex chromatography and Sepharose-6B gel filtration. A carboxylesterase associated with organophosphate resistance in the green bug, *Schizaphis graminum* was purified by column chromatography and preparative electrophoresis (Siegfried et al., 1997). In the present work, the purification of two carboxylesterases from the seeds of *Mucuna* is described.

Since one of the objectives of this study was the purification of pure homogenous esterases from the seeds of *M. pruriens* for further characterization, a simple reproducible method was established. The method involved extraction, ammonium sulphate fractionation, cation exchange chromatography on CM-cellulose, gel filtration on Sephadex G-100 and preparative PAGE. Results of the purification showing the recovery, fold purification and the specific activity at each stage are given in Table 1. The crude extract was subjected to fractional precipitation using ammonium sulphate, change of pH and chilled acetone. Considerable loss of esterase activity was observed with change in pH and addition of acetone. On the other hand, ammonium sulphate fractionation gave a good yield with an increase in fold purification. Hence, ammonium sulphate precipitation was selected for fractionation of esterases from the crude extract.

The ammonium sulphate fraction was subjected to cation exchange chromatography using CM-cellulose. The elution profile of CM-cellulose chromatography is shown in Fig. 1a. Three peaks of esterase activity were eluted and designated as fraction I, fraction II and fraction III. Fraction I was not adsorbed onto the column and hence eluted with the starting buffer. Fraction II was eluted by 0.1 M sodium chloride and fraction III by 0.3 M sodium chloride in starting buffer. The fractions II containing an appreciable amount of esterase activity were pooled and then concentrated using ammonium sulphate.

The concentrated CM-cellulose fraction II was subjected to gel filtration on Sephadex G-100, the elution profile is shown in Fig. 1b. The protein and the esterolytic activity were eluted in one peak. Native PAGE of peak fractions from Sephadex G-100 chromatography, after staining for esterases, showed two esterolytic bands. The Sephadex G-100 fraction was subjected to preparative PAGE and the two esterolytic bands were isolated and designated ME-III and ME-IV. ME-III was purified to about 31-fold with a recovery of 6.11% and ME-IV was purified to about 51-fold with 9.93% recovery.

2.2. Criteria of purity

Many researchers reported the homogeneity of their preparations using PAGE (Nourse et al., 1989). Carboxylesterases have been purified to homogeneity and purity was established by both gel electrophoresis and isoelectric focussing (IEF) (Upadhya et al., 1985). The homogeneity of the carboxylesterase isolated from the latex of *S. grantii* was established by PAGE, IEF and SDS–PAGE (Govindappa et al., 1987). In the present investigation, PAGE, IEF and SDS–PAGE have been employed to establish the homogeneity of the *M. pruriens* esterases, ME-III and ME-IV. PAGE of ME-III and ME-IV showed a single esterase band (Fig. 2a and b) and corresponding protein band (Fig. 2c and d). IEF of ME-III and ME-IV showed a single esterase band and a corresponding protein band (Fig. 3a and b). SDS–PAGE in the presence and absence of β-mercaptoethanol and showed single protein bands, suggesting the monomeric nature of the *Mucuna* esterase enzymes (Fig 4a and b).

2.3. Isoelectric pH

The isolelectric pH values of purified *Mucuna* seed carboxylesterases, ME-III and ME-IV were 6.8 and 7.4 and they showed binding affinity to a cation exchanger at pH 5.5. The near basic isoelectric pH values and binding affinity to a cation exchanger probably indicate the presence of large proportions of basic amino acids in these enzymes. However, most of the carboxylesterases studied so far in both animals and plants have low isoelectric pH values and contain large proportions of acidic amino acids. The purification of carboxylesterases in most of the cases involved the use of anion exchangers (Upadhya et al., 1985; Govindappa et al., 1987; Sreerama et al., 1991). The low isoelectric pH values, the presence of large proportions of acidic amino acids and binding affinity to the anion exchanger clearly indicate the acidic nature of carboxylesterases in many cases. Eric Haubruge (2002) reported carboxylesterases of isoelectric pH values of 7.3 and 6.6 from malathion resistant and susceptible insects, *Tribolium castaneum*.

Table 1
Purification of esterases from soaked seeds of *Mucuna pruriens.*

Purification step	Total Volume (ml)	Total protein (mg)	Total activity (μ mole/min)	Specific* activity	Fold purification	% Yield
Crude	175	1078	52.36	0.0486	1	100
40–80% ammonium sulphate fractionation	60	765.6	43.49	0.0568	1.168	83
CM-cellulose Fraction-II	70	70.664	28.69	0.406	8.353	54.79
Sephadex fraction	12	10.984	10.2	0.928	21.65	16.46
Preparative PAGE -Fraction II (ME-III)	4	2.1	3.2	1.52	31.27	6.11
Preparative PAGE -Fraction I (ME-IV)	4	2.1	5.2	2.48	51.03	9.93

* μ mole/min/mg protein.

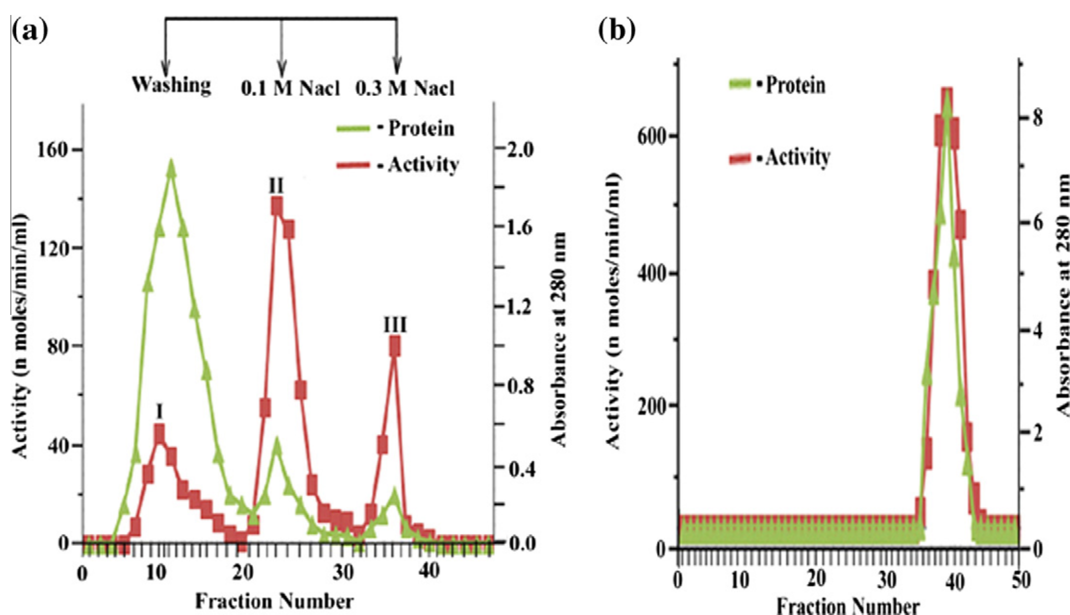

Fig. 1. (a) Elution profile of esterases from the soaked seeds of *Mucuna pruriens* on CM cellulose using 0.025 M sodium acetate buffer pH 5.5 The proteins were eluted by stepwise increase in the ionic strength using 0.1 and 0.3 M sodium chloride (b) Elution profile of CM-cellulose fraction-II from the soaked seeds of *Mucuna pruriens* on Sephadex G-100 (1.13 × 100 cm).

2.4. Molecular weight

The molecular weights of ME-III and ME-IV carboxylesterases of *M. pruriens* seeds as determined by SDS–PAGE were found to be 21 kDa and 30.2 kDa (Fig. 4a and b), respectively, both in the presence and absence of 2-mercaptoethanol. This clearly indicates the monomeric nature of both the carboxylesterases. The monomeric nature of the purified *Mucuna* seed carboxylesterases (ME-III and ME-IV) was further confirmed by gel-filtration on Sephadex G-200 which indicated molecular weights of 20.89 and 31.62 kDa, respectively. The Stokes radii determined from the Porath's plot (1963) were 2.4 nm for ME-III and 2.7 nm for ME-IV. It is reported that most of the plant carboxylesterases have low molecular weights and contain a single polypeptide chain. The carboxylesterases from finger millet (Upadhya et al., 1985) and sorghum (Sae et al., 1971) were reported to have molecular weights of 60 to 70 kDa and found to contain a single polypeptide chain. Bartley and Stevens (1981) characterized and reported four carboxylesterases, each containing a single polypeptide chain, with molecular weights in the range of 35–50 kDa from apple. The carboxylesterase purified from the latex of *S. grantii* had a molecular weight of 14 kDa and consisted of single polypeptide chain (Govindappa et al., 1987). Two carboxylesterases each consisting of a single polypeptide chain with molecular weights of 23.5 and 30.2 kDa were isolated from the seeds of *J. curcas* L. (Staubmann et al., 1999).

2.5. Catalytic properties

2.5.1. Effect of time and enzyme concentration

The velocities of the enzyme catalyzed reaction with time were analyzed and the activity was linear up to 45 min for both the carboxylesterases. The effect of enzyme concentration on the hydrolysis of α-naphthyl acetate by purified carboxylesterases of *M. pruriens* was analyzed and the linearity was maintained up to 120 μg of protein for both the carboxylesterases.

2.5.2. Substrate specificity

Both the enzymes hydrolyzed α-naphthyl esters. ME-III and ME-IV exhibited specific activities of 1.48 and 4.16 μmole/min/mg protein for 1-naphthyl acetate and 2.45 and 7.53 for 1-naphthyl propionate, respectively. However, with acetylthiocholine chloride as substrate both did not show any activity. The physiological substrate of these carboxylesterases is not known and only naphthyl esters were used in the present investigation. Substrate specificity studies of purified fractions of bean, pea and finger millet carboxylesterases with phenyl esters, glyceryl esters and naphthyl esters (Montgomery et al., 1968; Veerabhadrappa and Montgomery, 1971; Upadhya et al., 1985) revealed that in all these cases, esterases hydrolysed short chain esters exhibiting preferential action towards propionyl esters. On the other hand, the rate of ester hydrolysis catalyzed by partially purified apple esterases increased with increase in the carbon number of the substrate in the order

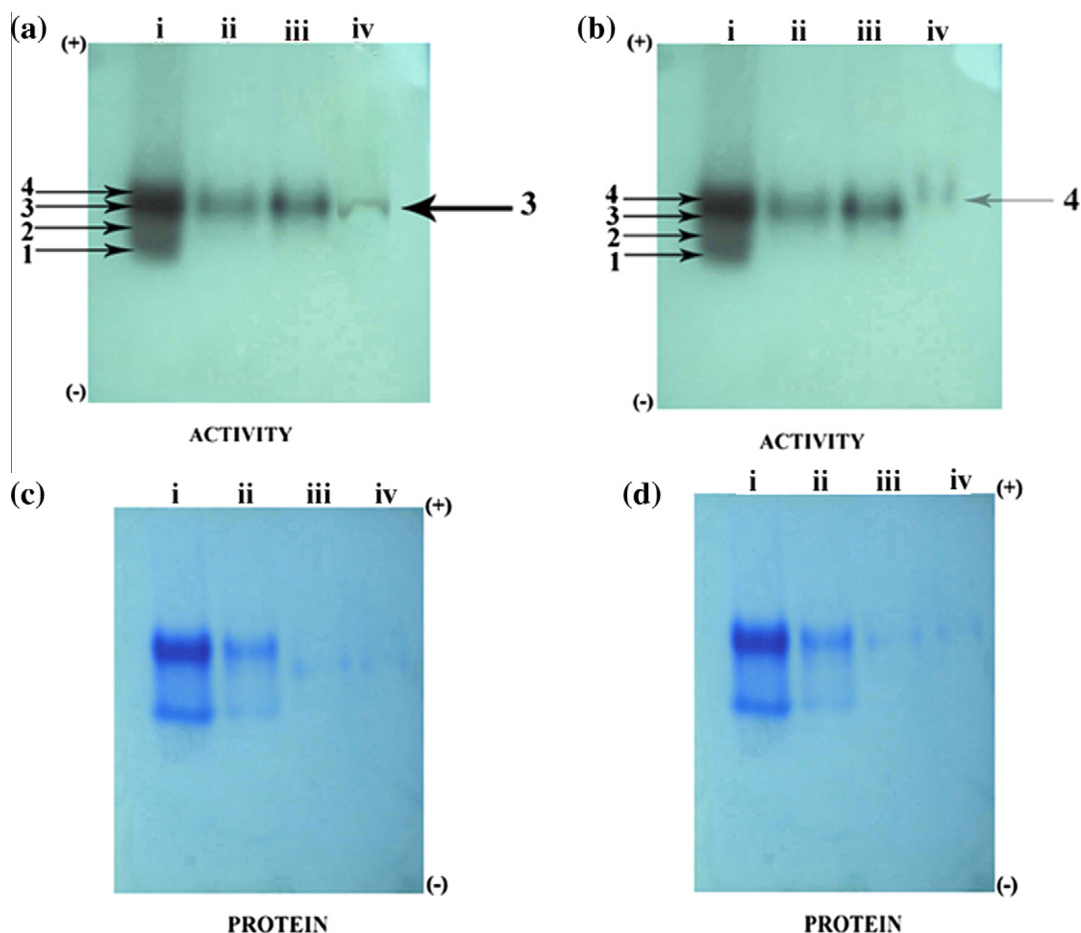

Fig. 2. (a–d) Native PAGE pattern of *Mucuna* seed esterase and proteins of III and IV. (i) Ammonium sulphate. (ii) CM-cellulose fraction-II. (iii) Sephadex fraction. (iv) Preparative PAGE fraction-II.

C_2–C_6, but activity declined with higher molecular weight esters. This activity was interpreted to be responsible for the hydrolysis of carboxylic acid esters during the ripening of apples (Bartley and Stevens, 1981). In case of finger millet esterases, Upadhya et al. (1985) reported that millet esterases showed more affinity towards the short chain naphthyl esters and the preferred substrate was naphthyl propionate. This activity was interpreted to be responsible for the hydrolysis of short chain fatty acid esters during the germination of the seeds. In the present investigation, *M. pruriens* esterases also showed more affinity towards the short chain naphthyl esters and among these, the preferred substrate was naphthyl propionate. This activity may be important for the hydrolysis of short chain fatty acid esters during growth and development of the plant.

2.5.3. Optimum pH and pH stability

The optimum pH obtained for most of the purified carboxylesterases from animals ranged from pH 7.0–9.0. Among plant carboxylesterases, an optimum pH of 7.0 was obtained for sorghum and barley (Sae et al., 1971; Burger et al., 1970) and 7.5 for finger millet and *S. grantii* (Upadhya et al., 1985; Govindappa et al., 1987). An optimum pH 7.0 (ME-III) and 7.5 (ME-IV) was obtained for *Mucuna* seed esterases (Fig. 5a). Both the enzymes were stable between pH 4.0–9.0.

2.5.4. Optimum temperature and temperature stability

The purified *Mucuna* seed carboxylesterases, ME-III and ME-IV were found to be optimally active at 45 and 37 °C (Fig. 5b), respectively. Although no inactivation was observed at 60 °C, both the en-

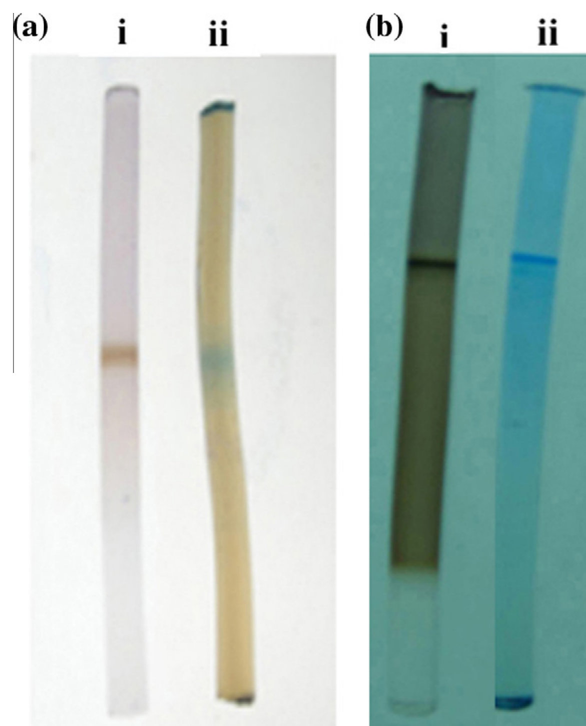

Fig. 3. (a and b) Gel electrofocussing pattern of purified *Mucuna* seed esterases, ME-III and ME-IV. (i) Activity (ii) Protein.

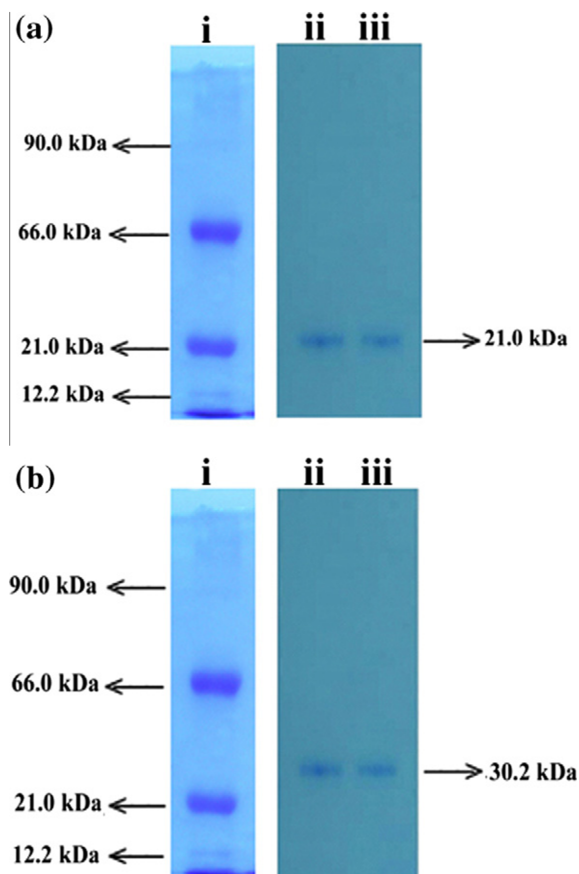

Fig. 4. (a) SDS–PAGE pattern of (i) standard proteins (ii) purified *Mucuna* seed esterase ME-III in the presence and (iii) absence of β-mercaptoethanol. (b) SDS–PAGE pattern of (i) standard proteins (ii) purified *Mucuna* seed esterase ME-IV in the presence and (iii) absence of β-mercaptoethanol.

zymes were completely inactivated at 65 °C. Both the enzymes were stable up to 60 °C. The energy of activation of ME-III is 15.9495 kJ/mole and that of ME-IV is 14.59 kJ/mole, respectively. The optimum temperature obtained for other plant carboxylesterases such as barley, finger millet, *S. grantii* and *Cucurbita pepo* was also in the above range (Burger et al., 1970; Upadhya et al., 1985; Govindappa et al., 1987; Fahmy et al., 2008).

2.5.5. K_m and V_{max}

The hydrolysis of naphthyl esters such as 1-naphthyl acetate and 1-naphthyl propionate catalyzed by *Mucuna* seed carboxylesterases ME-III and ME-IV follows typical Michaelis–Menten kinetics with no evidence of inhibition at high substrate concentrations. The K_m and V_{max} values were determined from Lineweaver–Burk plots (Fig. 6a and b). The K_m and V_{max} of ME-III for 1-naphthyl acetate are 0.10 mM and 6.40 n moles/min and for 1-naphthyl propionate are 0.052 mM and 9.4 n moles/min, respectively. Similarly, The K_m and V_{max} of ME-IV for 1-naphthyl acetate are 0.166 mM and 9.842 n moles/min and for 1-naphthyl propionate are 0.0454 mM and 13.0 n moles/min respectively. The analysis of K_m showed more affinity towards naphthyl propionate than naphthyl acetate. The purified fractions of bean, pea, finger millet and *S. grantii* carboxylesterases also showed similar types of substrate specificities exhibiting preferential action towards propionate esters (Montgomery et al., 1968; Veerabhadrappa and Montgomery, 1971; Upadhya et al., 1985; Govindappa et al., 1987). Further, the K_m and V_{max} values of the individual purified enzymes from the seeds of *Mucuna* revealed that each of them is a distinct species catalyzing the same reaction. Therefore, they can be considered as isoenzymes.

2.5.6. Inhibitor specificity and I_{50}

General esterases have been commonly classified into three types based on their interactions with inhibitors (Aldridge and Reiner, 1972). A-type esterases are not inhibited by organophosphates (OPs) but hydrolyze OPs, whereas B-type esterases are readily inhibited by OPs. In contrast, C-type esterases do not hydrolyze OPs nor are they inhibited by OPs. A subsequent elaboration of the Aldridge and Reiner (1972) scheme has been used to classify esterase isozymes detected after native PAGE and stained with various artificial ester substrates, in conjunction with organophosphate (OP), sulfhydryl, and the carbamate inhibitors (Holmes and Masters, 1967; Coates et al., 1975; Healy et al., 1991). The esterases are classified into four types. Carboxylesterases (EC 3.1.1.1), (Carboxyl ester hydrolase), are inhibited by OPs and prefer aliphatic esters, generally of longer fatty acid chain than acetate. Arylesterases (EC 3.1.1.2) (Aryl ester hydrolase) hydrolyze OPs and are inhibited by sulfhydryl inhibitors and generally prefer substrates with aromatic alcohol groups. Acetylesterases (EC 3.1.1.6) (acetic ester acetyl esterase) are not inhibited by any of these inhibitors and generally prefer substrates with acetyl groups. Cholinesterases are of two kinds, namely, (a) acetylcholinesterase (EC 3.1.1.7) (acetylcholine acetyl hydrolase) which is known as true or specific cholinesterase, and (b) cholinesterase (EC 3.1.1.8) (acetylcholine acyl hydrolase), also called as pseudocholinesterase, non-specific cholinesterase or butyrylcholinesterase. These esterases catalyze the hydrolysis of cholinesters at a higher rate than aliphatic and aromatic esters and are inhibited by OPs and carbamates. Based on

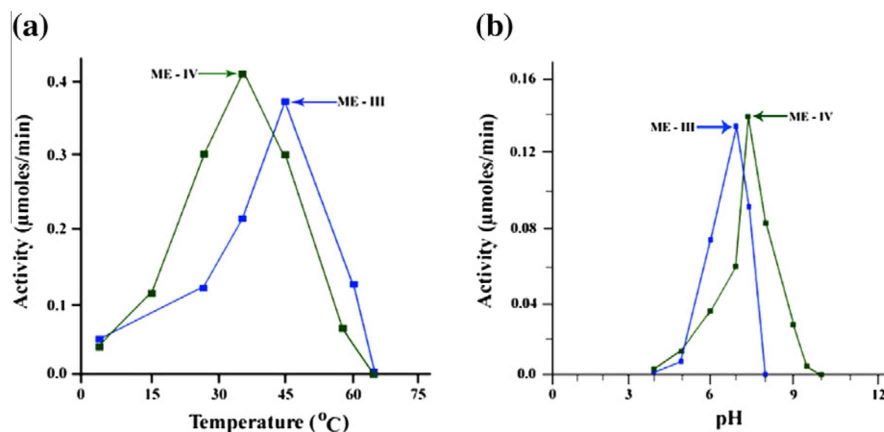

Fig. 5. (a) Activity of *Mucuna* seed carboxylesterases ME-III and ME-IV at different pH. (b) Activity of *Mucuna* seed carboxylesterases ME-III and ME-IV at different temperatures.

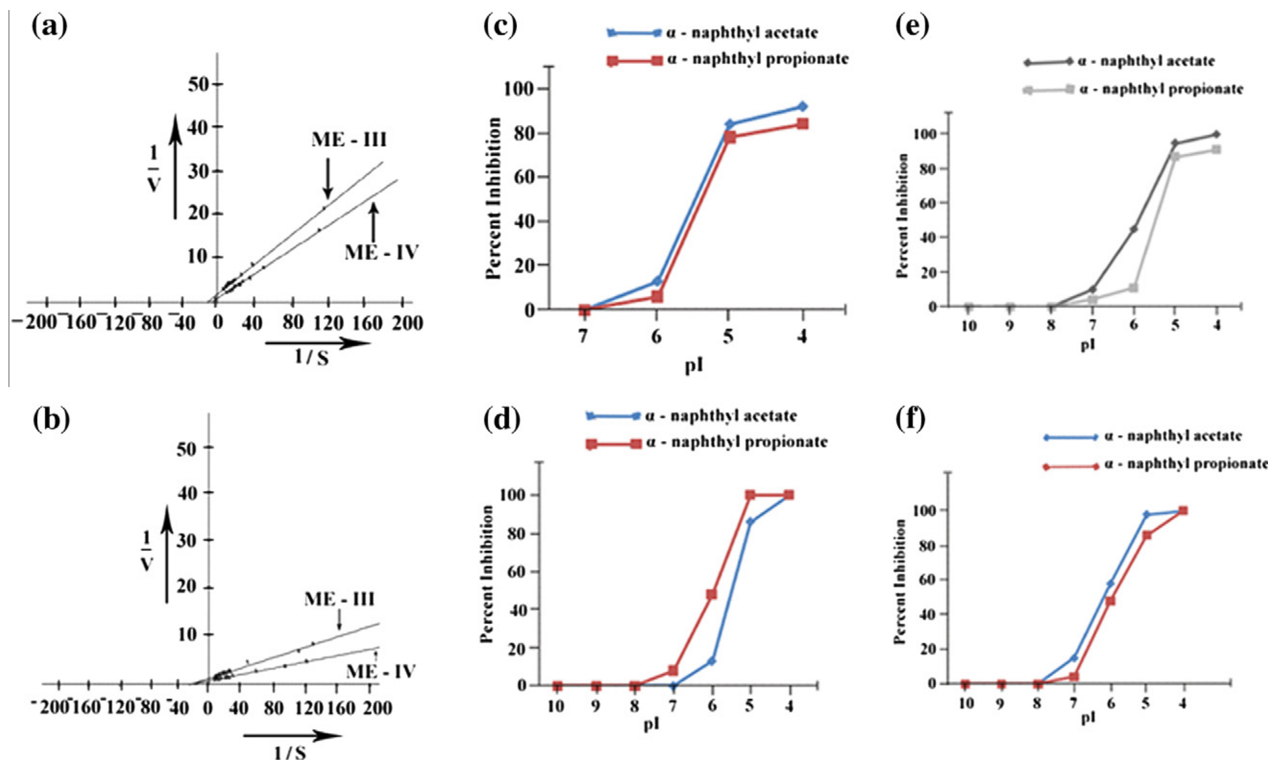

Fig. 6. (a) k_m and V_{max} determination of *Mucuna* seed carboxylesterases with 1-naphthyl acetate as substrate. (b) k_m and V_{max} determination of *Mucuna* seed carboxylesterases with 1-naphthyl propionate as substrate. (c) Inhibition curves for the hydrolysis of 1-naphthyl esters by *Mucuna* seed carboxylesterase ME-III in the presence of dichlorvos. (d) Inhibition curves for the hydrolysis of α-naphthyl esters by *Mucuna* seed carboxylesterase ME-III in the presence of phosphamidon. (e) Inhibition curves for the hydrolysis of α-naphthyl esters by *Mucuna* seed carboxylesterase ME-IV in the presence of dichlorvos. (f) Inhibition curves for the hydrolysis of α-naphthyl esters by Mucuna seed carboxylesterase ME-IV in the presence of phosphamidon.

Table 2
I_{50} of organophosphate inhibitors for carboxylesterases ME-III and ME-IV of *Mucuna pruriens*.

Carboxylesterase	Dichlorvos		Phosphamidon	
	1-Naphthyl acetate (M)	1-Naphthyl propionate (M)	1-Naphthyl acetate (M)	1-Naphthyl propionate (M)
ME-III	3.162×10^{-6}	3.98×10^{-6}	7.94×10^{-7}	3.162×10^{-6}
ME-IV	7.94×10^{-7}	3.162×10^{-6}	1×10^{-6}	1.584×10^{-6}

the above criteria, the two purified *M. pruriens* seed esterases (ME-III and ME-IV) separated by PAGE were stained with 1-naphthyl acetate in conjunction with OPs, carbamates and sulphydryl inhibitors. The esterases were inhibited only by OPs and hence were classified as carboxylesterases. Similar observations were noticed in case of insect carboxylesterases (Sreerama et al., 1991; Siddalinga Murthy et al., 1996) and plant carboxylesterases (Upadhya et al., 1985; Govindappa et al., 1987).

The plots of pI (inhibitor concentration) versus percent inhibition for both purified carboxylesterases with dichlorvos and phosphamidon are presented in Fig. 6c–f. Single sigmoid curves were obtained with both the organophosphate inhibitors tested. Dichlorvos and phosphamidon inhibited both the enzymes in the pI range 4 and 7. The I_{50} of dichlorvos and phosphamidon are tabulated in Table 2.

3. Conclusion

The carboxylesterases are a distinct group of enzyme molecules with overlapping substrate specificities and very little is known about their natural substrate. However, all esterases that have been studied in detail exhibited maximal activity towards esters containing a particular acyl group. The *Mucuna* seed esterases fall into a broad class of esterases that hydrolyze maximally shorter chain acyl esters. These enzymes exhibit higher activity towards short chain naphthyl esters and among them, the more preferred one was 1-naphthyl propionate. Furthermore, the marked inhibition by organophosphates, lack of inhibition by carbamates and *p*-chloromercuricbenzoate (PCMB) and inactivity towards the choline ester satisfies their classification as a carboxylesterases (EC 3.1.1). Further, carboxylesterases from the seeds of *M. pruriens* represented in this study have interesting characteristics such as, low molecular weight, high stability and high affinity towards short chain esters which they share with some plant and microbial esterases (Staubmann et al., 1999; Liu et al., 2001). Therefore, *M. pruriens* carboxylesterases may have a role in the hydrolysis of short chain fatty acid esters during plant growth and development.

4. Experimental

4.1. Plant material

The *Mucuna* seeds were collected from Siddarabetta, Tumkur district, Karanataka, India and Indian institute of horticulture research, Bangalore, India.

4.2. Chemicals

The ampholyte carrier (pH 3–10), acrylamide, *N*,*N* methylene bis acrylamide, CM-cellulose, Sephadex G-100, Sephadex G-200, ammonium per sulphate, 1-naphthyl acetate, Fast blue RR salt were purchased from Sigma chemicals company, St Louis, Missouri, USA. The inhibitors were gifts from Pesticides and Industrial Chemicals Repository, MD-8, Research Triangle Park, North California, USA. All other chemicals used were of analytical grade. The stock solutions of the inhibitors were prepared in acetone and diluted suitably with enzyme assay buffer.

4.3. Preparation of crude enzyme extract

The seeds from *M. pruriens* were collected, soaked and dehulled. The acetone powder from the dehulled seeds (10%) was prepared according to the method of Wetter (1957). A 10% extract of the acetone powder was prepared using 0.05 M sodium phosphate buffer pH 7.0 by stirring over a magnetic stirrer for 2 h at 4 °C and then centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant was collected.

4.4. Esterase 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 10–100 μg of enzyme was incubated at 27 °C for 15 min. The reaction was stopped by addition of 1 ml of DBLS reagent (2 parts of 1% diazo blue B and 5 parts of 5% sodium lauryl sulphate). In the control, enzyme was inactivated by DBLS prior to incubation with substrates. The absorbance of the developed color was measured at 600 nm. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of product per min at pH 7.0 and 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 eluents obtained from chromatographic columns was routinely monitored by measuring absorbance at 280 nm.

4.5. Polyacrylamide gel electrophoresis (PAGE)

Non-denaturing PAGE (7.5% T, 2.7% C) was performed at pH 4.3 according to the procedure of Reisfield et al. (1962). SDS–PAGE (10% T, 2.7% C) was performed after denaturing the proteins with SDS and β-mercaptoethanol. Gel-electrofocussing was performed by the method of Wrigley (1969), in 8% polyacrylamide gels. The electrophoresis was performed at 4 °C for 2 h. After the run, the gels were removed and stained for esterase activity as described earlier. The gels were stained for proteins using 0.02% coomassie brilliant blue G-250 (w/v) in 3.5% (w/v) perchloric acid and destained in distilled water.

4.6. Gel-localization of esterase activity

Esterase activity on polyacrylamide slab gels was detected by the method of Hunter and Markert (1957). The gels were stained for esterase activity after electrophoresis in a solution containing 100 ml of 0.05 M sodium phosphate buffer, pH 7.0, 40 mg of Fast blue RR and 20 mg of 1-naphthyl acetate (dissolved in 2 ml of acetone) for 20 min at 25 °C. Proteins were detected on the slab gels by staining with staining solution (1 g of coomassie brilliant blue R-250, 400 ml of methanol, 100 ml of glacial acetic acid and 500 ml of distilled water). The gels were destained using destaining solution containing 400 ml of methanol, 100 ml of glacial acetic acid and 500 ml of distilled water.

4.7. Purification

All the purification procedures were performed at 4 °C unless otherwise stated. To the crude extract, solid ammonium sulphate was added to 0–40% saturation at 4 °C. The precipitate obtained was removed by centrifugation at 10,000 rpm for 30 min. To the supernatant obtained, solid ammonium sulphate was added to 40–80% saturation at 4 °C. The precipitated protein was removed by centrifugation at 10,000 rpm for 30 min. The precipitate thus obtained was redissolved in 0.025 M sodium acetate buffer, pH 5.5 and dialyzed against the same buffer. The dialyzed fraction was loaded onto a CM-cellulose column (2.5 × 22 cm) pre-equilibrated in 0.025 M sodium acetate buffer, pH 5.5 at a flow rate of 30 ml/h. The bound proteins were eluted by stepwise increase in ionic strength using start buffer containing 0.1 and 0.3 M NaCl with a fraction volume of 10 ml. The CM-cellulose fraction II containing esterase activity were pooled, concentrated and applied to a Sephadex G-100 column (1.0 × 140 cm) pre-equilibrated with 0.025 M sodium phosphate buffer pH 7.0. The proteins were eluted with the same buffer and fractions of 2.0 ml were collected at a flow rate of 12 ml/h. The esterase enzymes eluted in a single peak. The fractions containing esterase activity were pooled, concentrated, dialyzed and subjected to preparative PAGE. The preparative polyacrylamide slab gel was prepared in 12 × 8 cm glass plates (0.3 cm thickness) as described earlier. The Sephadex G-100 fraction containing 6–8 mg of protein was loaded onto the gel and electrophoresed at 20 mA of current. After the electrophoresis, the gel was stained for esterase activity. The individual esterase activity bands formed were sliced and homogenized using glass homogenizer with chilled 0.025 M sodium phosphate buffer pH 7.0. The homogenates obtained were centrifuged at 3000 rpm for 15 min at 4 °C.

4.8. Molecular weight determination

The apparent molecular mass of the native enzymes were determined according to the method of Andrews (1970) using Sephadex G-200 (1.13 × 100 cm) pre-equilibrated with 0.025 M sodium phosphate buffer pH 7.0, at a flow rate of 10 ml/h. The column was calibrated using cytochrome-c (12.3 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (BSA) (66 kDa), alcohol dehydrogenase (150 kDa) and β-amylase (200 kDa). Blue Dextran (2000 kDa) was used to determine the void volume (V_o). The molecular weights of the carboxylesterases were determined from the plot of log molecular weight versus K_{av}. The molecular weights of the purified esterases were also determined by SDS–PAGE from the plot of log molecular weight against relative mobility.

4.9. Kinetic studies

Effect of time, effect of enzyme concentration, substrate specificity, optimum pH and pH stability, optimum temperature and temperature stability, K_m and V_{max} and inhibition studies were determined for the purified esterases.

4.10. Inhibitor specificity

The gels were preincubated with 1×10^{-5} M solution of OPs, carbamates and sulphydryl inhibitors for 30 min, washed with 0.05 M sodium phosphate buffer, pH 7.0 and stained for esterase activity as described in Section **4.5**.

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