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

K.S. Chandrashekharaiah [a,b,*], N. Ramachandra Swamy [b], K.R. Siddalinga Murthy [b]

[a] Department of Biotechnology, PES Institute of Technology, BSK III Stage, Hosakerahalli, Bangalore 560 085, Karnataka, India
[b] Department of Biochemistry, Central College Campus, Bangalore University, Bangalore 560 085, Karnataka, India

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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).

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-

* Corresponding author. Tel.: +91 80 26721983x344; fax: +91 80 26720886.
E-mail address: kschandraks@gmail.com (K.S. Chandrashekharaiah).

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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*.