J. Biosci., Vol. 12, Number 1, March 1987, pp. 71-86. © Printed in India.

Purification and characterisation of a carboxylesterase from the latex of *Synadenium grantii* Hook,'f'

T. GOVINDAPPA, L. GOVARDHAN, P. S. JYOTHY and P. S. VEERABHADRAPPA
Department of Chemistry, Central College, Bangalore University, Bangalore 560 001, India

MS received 4 September 1986; revised 29 January 1987

Abstract. The latex of *Synadenium grantii* was found to contain esterolytic activity. Polyacrylamide gel electrophoretic study coupled with substrate and inhibitor specificity studies revealed the presence of multiple forms of carboxylesterases and cholinesterases in the latex. One of the carboxylesterases of the latex was purified by acetone fractionation, carboxymethyl-Sephadex chromatography and Sepharose-6B gel filtration. The homogeneity of the enzyme was established by polyacrylamide gel electrophoresis, isoelectric focussing and sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The enzyme consists of a single polypeptide chain with a molecular weight of 14,000. The amino acid analysis of the purified enzyme revealed that it contained a greater number of neutral and acidic, compared to basic amino acid residues. The isoelectric pH of the enzyme was found to be 4·0. The enzyme was a glycoprotein as revealed by periodic acid Schiff-staining technique. Studies with different organophosphate and carbamate inhibitors showed that this enzyme was sensitive to organophosphates. The product inhibition studies with this enzyme showed linear competitive inhibition with acetate and linear non-competitive inhibition with 1-naphthol.

Keywords. Latex; *Synadenium grantii* Hook 'f'; carboxylesterases; cholinesterases; inhibitor specificity; purification and properties.

Introduction

The plant *Synadenium grantii* Hook 'f'; belongs to the family of Euphorbiaceae. It secretes a milky-white latex on breaking the twigs, which is highly toxic. The latex has been reported to contain proteolytic, haemagglutinin, fibrinolytic (Premaratna *et al.*, 1981) and phosphatase (Veeraswamy *et al.*, 1973-74) activities. Nothing has been reported about the latex esterases.

Esterases are a group of hydrolytic enzymes occurring in multiple molecular forms with broad substrate specificity. Though they have been extensively studied from living systems their exact physiological significance is not clearly known except for cholinesterases in animals. In the present investigation, characterisation of the esterases as well as purification and properties of a carboxylesterase present in *S. grantii* latex have been described.

Abbreviations used: PAGE., Polyacrylamide gel electrophoresis; BSA, bovine serum albumin; CM, carboxy methyl; PAS, periodic acid Schiff; SDS, sodium dodecyl sulphate; M_r, molecular weight; DTNB, 5,5'-dithiobis (2-nitrobenzoic acid); GLC, gas-liquid chromatography; PCMB, *p*-chloromercuribenzoate; K_i, bimolecular rate constant; I_{50}, inhibitor concentration to give 50% inhibition in enzyme activity.

Materials and methods

The latex was collected in a clean tube by breaking the tender twigs of the plant, diluted 20 fold with distilled water, mixed well and kept at room temperature for 30 min. This was then centrifuged at 1000 *g* for 15 min at 10°C. The clear supernatant was collected, chilled and cold acetone (—10°C) was added slowly with stirring to obtain 60% saturation. The precipitate formed was separated by centrifugation at 1000 *g* for 10 min, dissolved in 50 mM sodium phosphate buffer pH 7·0 and used for investigations.

Chemicals

All the chemicals used were of analytical grade or purchased from Sigma Chemical Co., St. Louis, Missouri, USA. The ampholyte carrier (pH 4-6) was obtained from Serva-Fein Biochemica, Heidelberg, Federal Republic of Germany. The inhibitors, dichlorvos (2,2-diethyl dichloro vinyl phosphate) and paraoxon (0,0-diethyl O-*p*-nitrophenyl phosphate) were gifts from Pesticides and Industrial Chemical Repository, MD-8, Research Triangle Park, North Carolina, USA and CIBA-GEIGY Ltd., Basel, Switzerland, respectively.

Solutions of inhibitors were prepared in a small volume of acetone and diluted suitably with 1% Triton X-100.

Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed essentially according to the method of Ornstein (1964) and Davis (1964). A discontinuous gel system consisting of a 7·5% separating gel and 3·0% spacer gel was used. The electrode buffer used was 0·03 M Tris-glycine, pH 8·3. An aliquot of the enzyme extract (200 μg protein) suitably diluted with 20% sucrose solution containing bromophenol blue, was carefully layered on to each gel and subjected to electrophoresis for 2 h at 10°C maintaining a current of 2 mA per gel tube.

Staining for esterase activity

Esterase activity was detected by the method of Hunter and Markert (1957). The gels were placed in 100 ml of 0·1 M phosphate buffer, pH 7·0 containing 40 mg Fast blue RR and 20 mg 1-naphthyl ester in 1 ml acetone, for 15 min at 37°C.

Staining for Cholinesterase activity

Cholinesterase activity was detected by the method of Karnowsky and Roots (1964). The gels were incubated, after electrophoresis, in 100 mM phosphate buffer, pH 7·0, containing 5mg of thiocholine ester, 0·5 ml of 100 mM sodium citrate, 1·0 ml of 30 mM copper sulphate and 1·0 ml of 5 mM potassium ferricyanide in a total volume of 10 ml. The white bands formed turned black on treatment with ammonium sulphide solution.

To study the effect of inhibitors, the gels were preincubated in the inhibitor solutions for 30 min at 27°C. At the end of the incubation period the gels were stained for esterase activity using the substrate-dye solution. A control gel was incubated in 1% Triton X-100 without inhibitor.

Enzyme assay

Carboxylesterase and acetylcholinesterase activities were monitored using 1-naphthyl esters (Gomori, 1953; Van Asperen, 1962) and thiocholine esters (Ellman *et al.,* 1961) as substrates, respectively. 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 37°C.

Protein was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard.

Purification

All operations were carried out at 5-6°C unless otherwise stated.

Carboxy methyl-Sephadex C-50 chromatography

The acetone precipitate was dissolved in 10 ml of 0·05 M phosphocitrate buffer pH 5·5 (starting buffer) and applied on to the carboxy methyl (CM)-Sephadex C-50 column (2×15 cm). The column was washed with 100 ml of the starting buffer. Thereafter, the proteins were eluted by stepwise elution using 150 ml each of starting buffers of pH 6·5 and 7·0. Fractions of 5 ml were collected at a flow rate of 30 ml per hour. Three protein peaks were obtained. The first peak fractions contained carboxyl-esterase activity, second peak fractions acetylcholinesterase activity and the third peak fractions no esterase activity.

Sepharose-6B chromatography

The first peak fractions of CM-Sephadex C-50 column were dialysed against distilled water and lyophilised. Then this fraction (approximately 1·0 ml) was applied onto Sepharose-6B column (1× 80 cm) previously equilibrated with the starting buffer of pH 7·0. The proteins were eluted with the same buffer at a flow rate of 10 ml/h and fractions of 2 ml were collected. Two protein peaks containing esterase activity were obtained. The fractions of the first peak were pooled, dialysed against distilled water and concentrated.

Protein bands in the gels were stained with 1% amido black in 6% acetic acid, and for glycoprotein the periodic acid Schiff (PAS) staining technique was followed (Rennert, 1967).

Molecular weight determination

Sodium dodecyl sulphate-PAGE method: Sodium dodecyl sulphate (SDS)-PAGE was carried out in 10% gels according to the method of Weber and Osborn (1969). Gel

electrofocussing was performed by the method of Wringley (1969) in 7·5% polyacrylamide gel (pH 4-6).

Gel filtration method: The molecular weight (M_r) of the purified enzyme was determined by the gel filtration method of Andrews (1964) using a Sephadex G-200 column (1 × 80 cm) equilibrated with 0·1 M phosphate buffer pH 7·0 and operated at a flow rate of 10 ml/h.

Amino acid analysis

The amino acid analysis of the enzyme was carried out according to the method of Moore and Stein (1963). The sample was hydrolysed at 110°C in 6 M HCl in an evacuated and sealed tube for 24 h. The amino acid analysis was done in a Beckman 121 MB automatic amino acid analyser. Tryptophan content of the protein was determined spectrophotometrically from the alkaline spectrum in 0·1 M NaOH by the method of Bencze and Schmid (1957). The free thiol groups in the protein were estimated by reaction with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) (Ellman, 1959).

Carbohydrate analysis

The method of Mckelvy and Lee (1969) was employed for the estimation of total sugar in the enzyme. Glucose (0–100 μg) was used in preparing the standard curve for total sugar estimation.

The method of Albersheim *et al.* (1967) was adopted for determination of the percentage composition of neutral sugars in the purified enzyme by gas-liquid chromatography (GLC) with a Varian Aerograph Series 1400 fitted with a flame-ionisation detector and a stainless-steel column (6' × 1/8") containing 3% of OV-225 on chromosorb-W (HP 80–100 mesh). Nitrogen was used as the carrier gas. GLC of alditol acetates was performed according to the method of Bjorndal *et al.* (1967).

Kinetic studies

The substrate and inhibitor specificity studies were carried out on the purified latex enzyme. Kinetic parameters such as K_m, V_{max} k_i, I_{so}, temperature and pH optima, and energy of activation were determined. In addition, temperature and pH stability studies were also carried out.

Results

Substrate specificity

The activity of the latex esterases with naphthyl esters and thiocholine esters is shown in figure 1. A total of 7 esterolytic bands were detected with 1-naphthyl acetate and 1-naphthyl propionate. These esterase bands based on their mobility

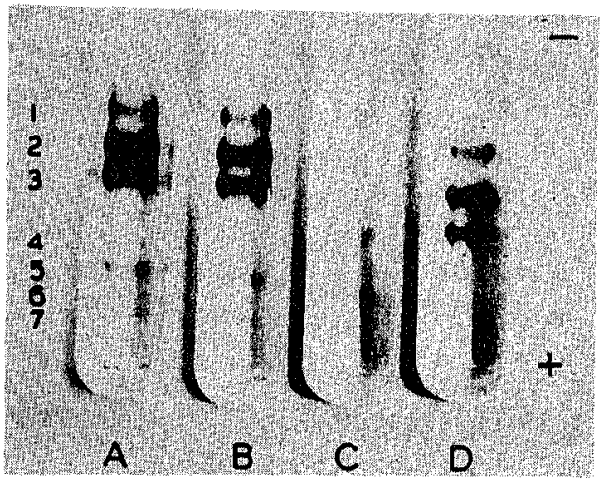

Figure 1. Esterase zymograms of *S. grantii* latex using different substrantes. (A), 1-Naphthyl acetate; (B), 1-naphthyl propionate; (C), 1-naphthyl butyrate; (D), acetylthiocholine iodide.

could be divided into two groups: the slow moving group (bands 1, 2 and 3) and the fast moving group (bands 4, 5, 6 and 7). The esterolytic bands of the slow moving group were more intense and active towards C_2-and C_3-esters of naphthol and thiocholine but were inactive with corresponding C_4-esters. It can be seen that C_2- and C_3-esters of naphthol and thiocholine were preferred substrates. The enzymes corresponding to bands 4–7 were shown to be active only with naphthyl esters and were not active towards thiocholine esters.

Inhibitor specificity

The effect of organophosphates (paraoxon and dichlorvos), carbamates (eserine sulphate, carbaryl and neostigmine bromide) and *p*-chloromercuribenzoate (PCMB) on the esterase activity using 1-naphthyl acetate as substrate is shown in figure 2.

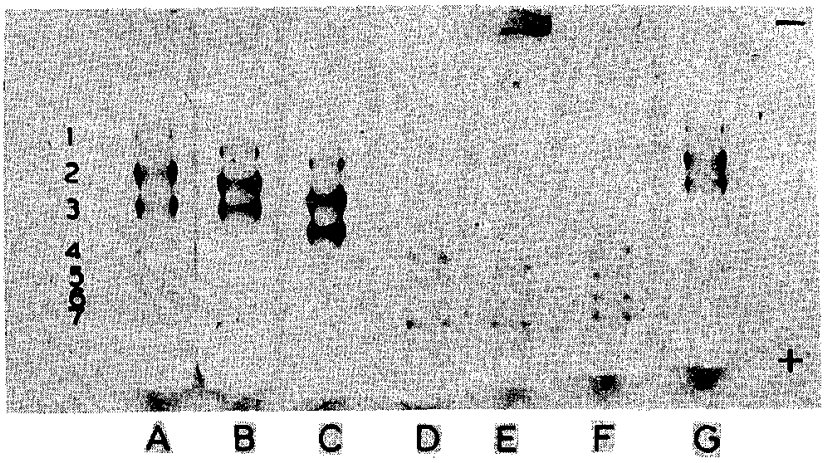

Figure 2. Esterase zymograms of *S. grantii* latex with 1-naphthyl acetate as substrate after incubation with different inhibitors. (A), No inhibitors; (B), dichlorvos (10^{-3} M); (C), paraoxon (10^{-3}M); (D), eserine sulphate (10^{-6}M); (E), carbaryl (10^{-6}M); (F), neostigmine bromide (10^{-6}M); (G), PCMB (10^{-3}M).

The organophosphates were found to inhibit the fast moving esterases at 10^{-3} M concentration. On the other hand, the carbamates were found to be effective inhibitors of the slow moving group esterases. Even at 10^{-6} M concentration the slow moving bands were inhibited but the bands of the fast moving group were not affected even at 10^{-3} M concentration of carbamates. Neither the slow moving nor the fast moving group of esterases were affected by PCMB.

Purification

Table 1 summarises the results of isolation and purification of the latex carboxylesterase. The gel electrophoretic study revealed that the 7 bands of esterolytic activity present in the crude enzyme were distributed in the first two peaks of the CM-Sephadex C-50 column (figure 3). Peak-I contained only carboxylesterase acti-

Table 1. Purification of carboxylesterase of *S. grantii* latex.

Purification step	Total volume (ml)	Total protein (mg)	Total units (μ mol/min)	Specific activity (units/mg)	Yield (%)	Purification
Aqueous extract	600	600	483	0·805	100	1
0–60% acetone fraction	40	280	445	1·59	92·1	2
CM-Sephadex C-50 chromatography						
CMS-I Fraction	20	40	200	5·0	41·4	6
CMS-II Fraction	150	25	230	9·2	47·6	11
Gel filtration of CMS-I Fraction on Sepharose-6B						
Sepharose-6B I Fraction	4	0·6	48·5	80·8	10·0	100

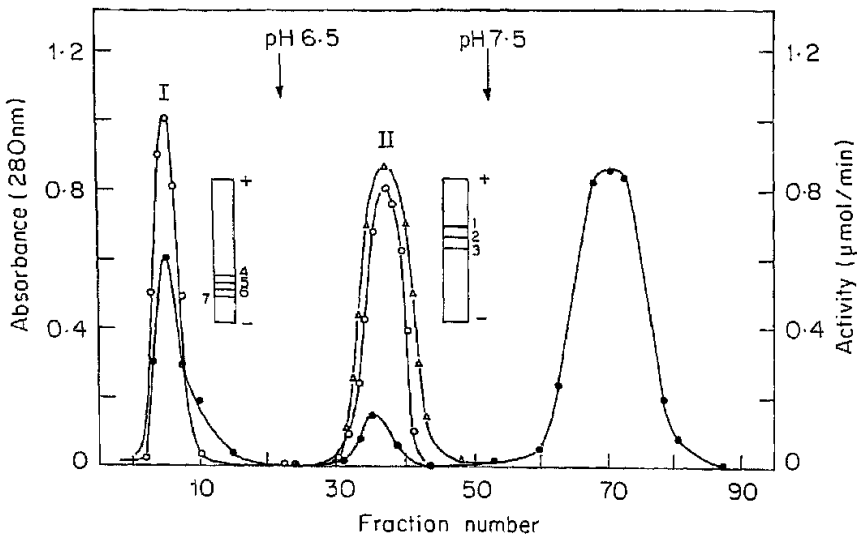

Figure 3. Elution pattern and electrophoretic zymograms of 60% acetone fraction of the *S. grantii* latex from CM-Sephadex C-50 column using stepwise elution. (○) and (△), esterase activity with 1-naphthyl acetate and acetylthiocholine iodide respectively; (●), protein (absorbance 280 nm).

vity (bands 4–7) and peak-II showed only acetylcholinesterase activity (bands 1-3). The elution profile of the CM-Sephadex peak-I fraction from the gel filtration on Sepharose-6B column is shown in figure 4. Carboxylesterase corresponding to band 7 was present in peak-I while the remaining bands along with some non-esterolytic proteins were present in peak-II. Peak-I carboxylesterase was purified to about 100 fold with a recovery of 10%.

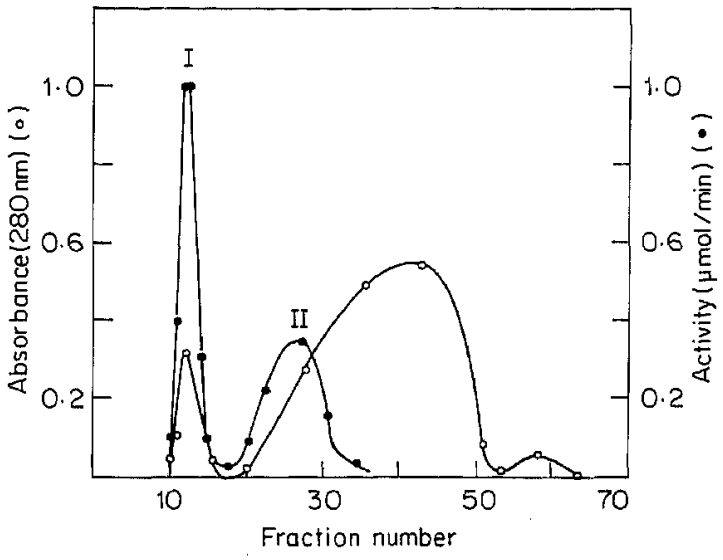

Figure 4. Sepharose-6B gel permeation chromatography of CMS-I fraction. (●), Esterase activity; (○), protein (absorbance 280 nm).

Criteria of homogeneity

The homogeneity of the Sepharose-6B peak-I fraction was tested by PAGE-method. The gel patterns obtained following esterase staining and protein staining are shown in figure 5A. A single band in both the patterns was seen. Isoelectric focussing of the purified enzyme resulted in a single esterase band (figure 5B) corresponding to a pI of 4·0. The purified enzyme also showed only one protein band on subjecting it to SDS-PAGE both in the presence and absence of 2-mercaptoethanol as shown in figure 5C. All these results indicate the homogeneity of the carboxylesterase.

M_r

The M_r of the carboxylesterase estimated by SDS-PAGE technique was found to be 14,000 (figure 6A), while gel filteration on Sephadex G-200 gave a M_r very much larger than the one obtained by SDS-PAGE method, as the esterase was eluted in the void volume (figure 6B).

Amino acid composition

Table 2 gives the results of the amino acid analysis of the esterase, expressed as

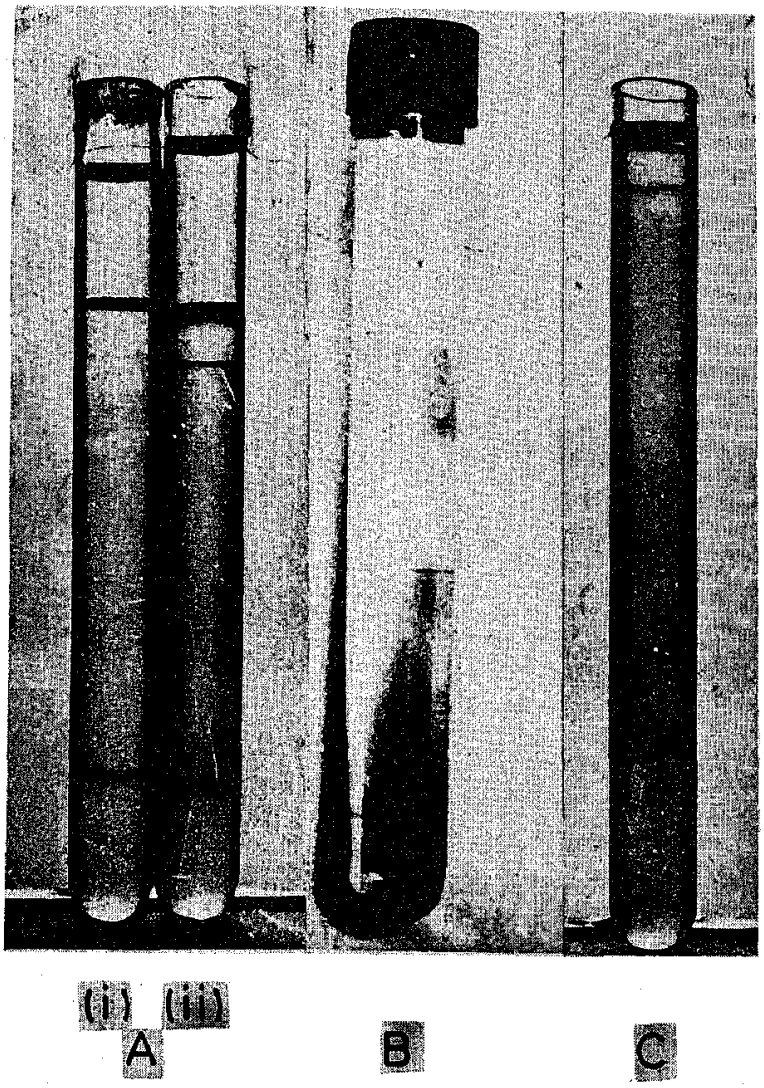

Figure 5. **A**. PAGE of Sepharose 6B-I fraction, (i) Protein staining; (ii) activity staining.. **B.** Esterase staining of Sepharose 6B-I fraction after electrofocussing. **C.** SDS-PAGE of Sepharose 6B-I fraction in 10% gel. The gel was stained with Coomassie brilliant blue-G with and without mercaptoethanol. Direction of migration is from top (cathode) to bottom (anode).

residues/mol of the enzyme. The enzyme was found to be composed of 132 amino acid residues per mol. The enzyme contained less of half-cystine, methionine, tyrosine, tryptophan, lysine and histidine residues but had relatively more neutral and acidic amino acid residues. It did not show the presence of any free thiol groups as indicated by a negative test for free thiol groups with DTNB.

Carbohydrate composition

The purified carboxylesterase was found to contain 24% neutral sugars. The percentage composition of arabinose (52·2), glucose (20·2) and mannose (17·6) was found to be relatively more compared to xylose (6·97) and galactose (3·0).

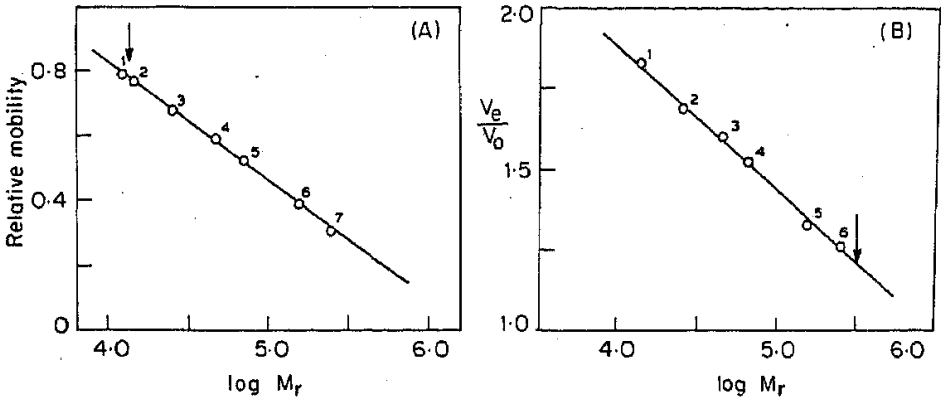

Figure 6. M_r determination of the latex carboxylesterase. **A.** By SDS-PAGE—The marker proteins used were (1) cytochrome-c; (2) lysozyme; (3) α-chymotrypsinogen; (4) ovalbumin; (5) BSA-monomer; (6) immunoglobulin G; (7) catalase. **B.** By gel filtration on Sephadex G-200. The marker proteins used were (1) cytochrome-c; (2) α-chymotrypsinogen; (3) ovalbumin; (4) BSA-monomer; (5) immunoglobulin G; (6) catalase. Arrow corresponds to the latex carboxylesterase.

Table 2. Amino acid composition of carboxylesterase of *S. grantii* latex.

Amino acid	Residues/mol	Nearest integer
Asp	14·82	15
Thr	6·87	7
Ser	9·72	10
Glu	11·64	12
Pro	6·32	6
Gly	11·61	12
Ala	10·54	11
Half-Cys	2·4	2
Val	6·41	6
Met	1·88	2
Ile	7·18	7
Leu	11·25	11
Tyr	8·23	8
Phe	7·01	7
Lys	6·03	6
His	1·00	1
Arg	2·59	3
*Trp	5·6	6
		132 residues

*Tryptophan was estimated spectrophotometrically from the alkaline spectrum.

Catalytic properties of the enzyme

The optimum pH and temperature for the enzyme were 7·5 and 45°C, respectively. The energy of activation of the enzyme was 12·6 Kcal/mol for hydrolysis of 1-naphthyl acetate. The enzyme was assayed at varying concentrations of 1-naphthyl esters and from the L-B plots the K_m and V_{max} values were determined (1-naphthyl

acetate, $7{\cdot}14 \times 10^{-4}$M and $0{\cdot}4\mu$ mol; 1-naphthyl propionate, $1{\cdot}67 \times 10^{-4}$M and $0{\cdot}6\mu$ mol; 1-naphthyl butyrate, 4×10^{-4}M and $0{\cdot}32\mu$ mol).

Temperature and pH stability

The temperature stability of the enzyme was studied by incubating the enzyme at different temperatures from 40° to 70°C for different intervals of time followed by rapid cooling to 0°C. The enzyme was then assayed at the optimum temperature of 45°C. Although no inactivation was observed at 45°C for atleast 2 h there was progressive inactivation with increase in temperature and complete inactivation at 70°C. The relationship between temperature and K_m or V_{max} is shown in figure 7. The enzyme was found to be stable between pH 5 and 7·5.

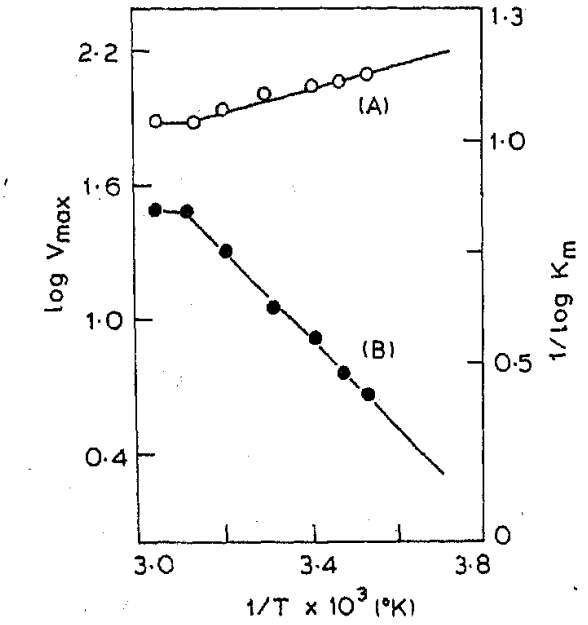

Figure 7. Effect of temperature on K_m and V_{max} of the latex carboxylesterase. Log $1/K_m$ (A) and log V_{max}(B) are plotted against $1/T \times 10^3$(K).

Product inhibition

Product inhibition studies were carried out by incubating the enzyme with a constant concentration of the product (either acetic acid or 1-naphthol) for 30 min. A competitive type of inhibition was obtained with acetic acid (figure 8A) and a non-competitive type of inhibition was obtained with 1-naphthol (figure 9A). A replot of the slopes of the lines *vs* concentration of acetic acid and 1-naphthol respectively gave straight lines (figures 8B and 9B).

Inhibition by organophosphates

A kinetic study of the esterase with paraoxon and dichlorvos was made. The

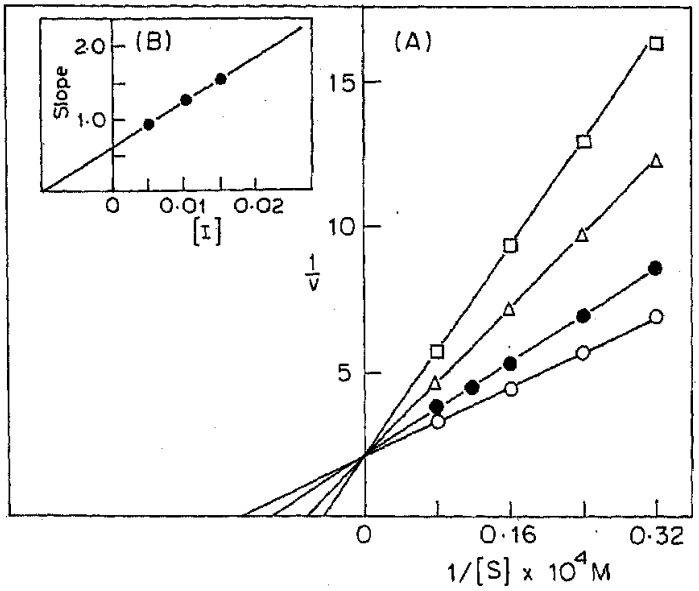

Figure 8, A. Lineweaver-Burk plots for the latex carboxylesterase using 1-naphthyl acetate as substrate in the presence of acetic acid. (●) with 0·005 M; (△) with 0·01 M; (□) with 0·015 M; (○) without acetic acid. **B.** Replot of slopes *vs* acetic acid concentrations.

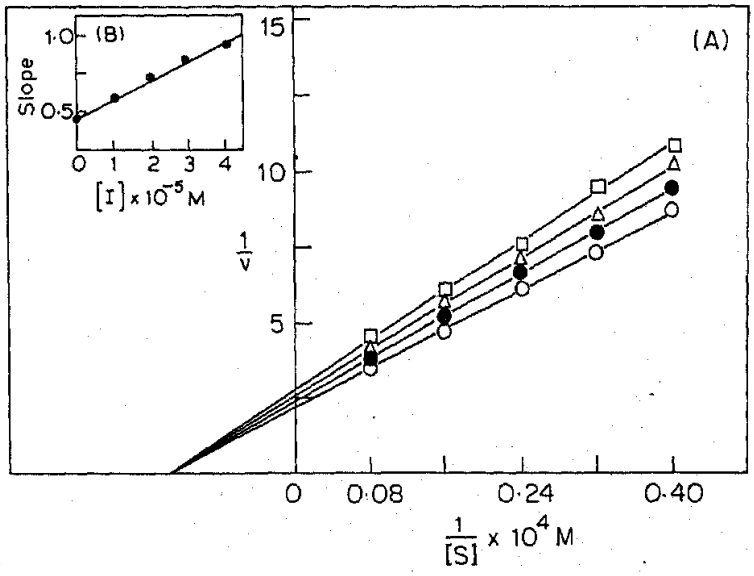

Figure 9. A. Lineweaver-Burk plots for the latex carboxylesterase using 1-naphthyl acetate as substrate in the presence of 1-naphthol (●) 1×10^{-5}M; (△) 2×10^{-5}M; (□) 3×10^{-5}M; (○) without 1-naphthol. **B.** Replot of slopes *vs* 1-naphthol concentration.

inhibitor rate constant k_i was determined according to the method of Aldridge (1950). The enzyme was incubated for different time intervals with inhibitors prior to the addition of substrate. The results obtained were plotted as log per cent activity *vs* time (figure 10). The bimolecular rate constant (k_i) for the reaction, $E+I\rightarrow EI$ was calculated from the equation, slope=$k_i(I)$/2-303 and the I_{50} values from the equation, $I_{5O} = 0{\cdot}693/k_i t$. k_i and I_{50} values were $0{\cdot}767\times10^5$ min^{-1} Mol^{-1} and 9×10^{-4} M re-

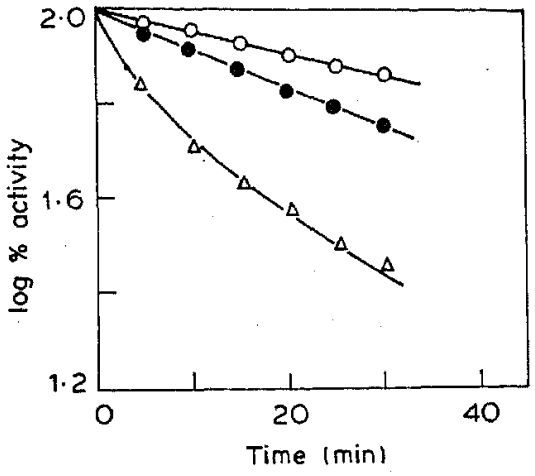

Figure 10. Effect of time on the inhibition of latex carboxylesterase by dichlorvos and paraoxon. (○) 1×10^{-6}M dichlorvos; (△) 1×10^{-4}M dichlorvos; (●) 1×10^{-6}M paraoxon.

spectively for dichlorvos and $1{\cdot}44 \times 10^{5}$ min^{-1} Mol^{-1} and $4{\cdot}816 \times 10^{-4}$ M, respectively for paraoxon.

The results of another set of experiments in which the enzyme was added to a mixture of substrate and inhibitor without preincubation of the enzyme with inhibitor is given in table 3.

Table 3. Effect of the inhibitor (DDVP) on the latex carboxylesterase.

Concentration of I (M)	Inhibition (%) (A)	(B)
10^{-3}	100	90
10^{-4}	95	80
10^{-5}	70	50
10^{-6}	40	20
10^{-7}	10	5
10^{-8}	0	0

(A) Enzyme was preincubated for 30 min with 'I' and then 'S' and 'I' mixture was added.
(B) Enzyme was added to the mixture of 'S' and 'I' directly.

The enzyme was incubated with different concentration of dichlorvos and paraoxon in the presence of 0·05 M phosphate buffer pH 7·0 for 30 min prior to the addition of substrate, which also contained the inhibitor to prevent dilution of the inhibitor in the enzyme. Straight line plots were obtained for both the inhibitors over a wide range of inhibitor concentration.

Discussion

Preliminary studies of the latex obtained from *S. grantii* indicated the presence of 7 enzyme species having esterase activity. In the present study, of the 7 esterolytic

bands exhibiting activity with C_2-and C_3-naphthyl esters, only the bands belonging to the slow moving group (bands 1-3) hydrolysed C_2-and C_3-thiocholine esters. Thus they may be classified as cholinesterases. Further, the absence of activity with C_4-esters of thiocholine and 1-naphthol, a property used to distinguish these enzymes from pseudocholinesterases (Augustinsson, 1948; Oosterbaan and Jansz, 1965), indicates that these enzymes are acetylcholinesterases. On the other hand, the enzymes belonging to the fast moving group are believed to be carboxylesterases as these are not active towards thiocholine esters. Since the esterases possess overlapping substrate specificity, the classification based mainly on substrate specificity alone needs further confirmation. Applying the criteria of classification of esterases (Holmes and Masters, 1967), the slow moving group of enzymes which were completely inhibited by carbamates and affected the least by organophosphates were categorised as acetylcholinesterases (EC 3·1·1·7). The enzyme classified as acetylcholinesterase from pea seedlings (Kasturi and Vasantharajan, 1976), bean seedlings (Ernst and Hartmann, 1980) and ragi seeds (Veerabhadrappa and Upadhya, 1979) was found to exhibit similar properties. The fast moving group of enzymes possessing no cholinester hydrolysing activity and sensitive only towards organophosphates were characterised as carboxylesterases (EC 3·1·1·1) as in the case of ragi seeds (Veerabhadrappa and Upadhya, 1979), soybean (Payne and Koszykowski, 1978), sorghum grain (Sae *et al.*, 1971), wheat (Macko *et al.*, 1967; Batia and Nelson, 1969), carrot (Carino and Montgomery, 1968) and potato (Desborough and Peloquin, 1968).

Of the 7 esterases of the latex, one has been purified to homogeneity. Several lines of evidence indicate that the purified esterase is a carboxylesterase. This carboxylesterase exhibits complete inhibition with organophosphates and is active towards short chain carboxylic esters. The enzyme does not hydrolyse the cholinesters tested. Similar observations were reported in the case of finger millet carboxylesterase (Upadhya *et al.*, 1985) and insect esterase (Veerabhadrappa *et al.*, 1980).

The M_r of the latex esterase was estimated to be around 14,000 by SDS-PAGE method and by amino acid analysis. Most of the carboxylesterases isolated and purified from animal and insect sources were reported to possess high M_r and were oligomeric in nature consisting of two or more subunits (Krisch, 1972). Unlike these enzymes, the latex esterase is a fairly low M_r protein, consisting of a single polypeptide chain. This is in agreement with other plant carboxylesterases whose M_r are in the range 40,000 to 70,000 (Upadhya *et al.*, 1985; Bartley and Stevens, 1981; Sae *et al.*, 1971). It should be noted that the latex esterase behaved as a very high M_r protein during gel filtration on Sephadex G-200 and Sepharose-6B eluting in the void volume. A similar observation was made with human pancreatic juice carboxylesterase (Lambardo *et al.*, 1978) for which the M_r was reported to be 100,000 as determined by SDS-PAGE, ultracentrifugation and amino acid analysis data. However, it was assigned a M_r around 300,000 by gel filtration on Sephadex G-200. This abnormal behaviour was attributed to the peculiar structure of the enzyme during gel filtration and not because of any aggregation as the elution was carried out with high ionic strength buffer. In the case of the latex carboxylesterase the elution pattern was the same irrespective of the ionic strength of the buffer. Hence, further studies are needed to account for the anamolous behaviour exhibited by this enzyme during gel filtration.

The isolated latex carboxylesterase contains more aspartic and glutamic acid

residues accounting for the acidic nature of the protein as evidenced by its electrophoretic mobility and its low pI value. Similar is the case with the plant esterases (Upadhya *et al.,* 1985) and animal carboxylesterases (Scott and Zerner, 1975; Axenfors *et al.,* 1979; Lambardo *et al.,* 1978) isolated so far.

The effect of temperature on the K_m and V_{max} values indicates that some conformational change in the latex enzyme protein may occur at the transition temperature, 45°C, as suggested for the carboxylesterases isolated from *Bacillus stearothermophilus* (Iwai *et al.,* 1983) and *Aspergillus niger* (Okumara, *et al.,* 1983). Linear Arrhenius plot obtained for the latex carboxylesterase indicates the presence of only one enzyme form unlike in the case of cockroach esterases (Matsunaga *et al.,* 1974) where nonlinear Arrhenius plots were noticed, which was attributed to the presence of more than one enzyme form in the preparation.

The enzyme showed more affinity towards short chain naphthyl esters and, among these, the preferred one was the propionate ester (low K_m and high V_{max}). Similar substrate specificities were exhibited by the purified preparations from bean, pea and finger millet esterases (Montgomery *et al.,* 1968; Veerabhadrappa and Montgomery, 1971; Upadhya *et al.,* 1985). The kinetic data obtained for the latex esterase with the products were consistent with the ordered release of products by the enzyme, alcohol being the first and acetic acid being the next to be released (Hofstee, 1960). The random release of products was ruled out in the present investigation. The product inhibition pattern also agreed with the Uni-Bi-Kinetic scheme with alcohol as the leading product.

Determination of the bimolecular rate constant, k_i is generally considered to be the most reliable criterion (O'Brien, 1960) by which the inhibitory power of an organophosphate for an esterase can be ascertained. The rate constant k_i for both dichlorvos and paraoxon was calculated from the linear plots and showed that paraoxon was a more effective inhibitor for the latex esterase. Upadhya *et al.* (1985) have reported a higher value of k_i with dichlorvos for the finger millet carboxylesterase. A linear relationship was obtained for the latex enzyme between the log per cent activity and the time of incubation, at relatively lower concentration of organophosphates. However, non-linear first order kinetics was observed at higher concentrations of dichlorvos (figure 10). The reason for this may be the unstable nature of the phosphorylated enzyme or the destruction of the inhibitor either enzymatically or non-enzymatically (Veerabhadrappa *et al.,* 1980; Aldridge and Reiner, 1972). The rate constant k_i obtained for the irreversible inhibition of the latex enzyme by organophosphates was higher compared to finger millet carboxylesterase (Upadhya *et al.,* 1985) indicating that this enzyme is more susceptible to these inhibitors. This is also in agreement with lower values of I_{50}. The results obtained when the enzyme was added to a mixture of substrate and inhibitor without any preincubation of the enzyme with the inhibitor, indicate that the presence of substrate partially protected the enzyme from inhibition (table 3).

Linear inhibition kinetics was obtained for the latex carboxylesterase with organophosphates when log per cent activity was plotted against inhibitor concentration. With most of the purified esterases from insects (Matsunaga *et al.,* 1974) and other animal tissues (Barker and Jencks, 1969; Levy and Ocken, 1969) non-linear inhibition kinectics was obtained when log per cent activity was plotted against organophosphate inhibitor concentration. This was attributed to a reversible aggregated enzyme system with allosteric behaviour. The linear first order inhibition

kinetics exhibited by the latex carboxylesterase negates the possibility of molecular aggregation.

The esterases are an ill-defined 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 latex esterase, although not exhaustively analysed, falls into a broad class of esterases that hydrolyse maximally shorter chain acyl compounds. This enzyme exhibits higher activity towards short chain naphthyl esters. Furthermore, the marked inhibition by organophosphates and lack of inhibition by carbamates and *p*-chloromercuribenzoate (PCMB) on the activity of the esterase satisfies its categorisation as a carboxylesterase (EC 3·1·1·1). Further investigation on the identification of the physiological substrate may throw light on its biological function in the latex.

Acknowledgements

We wish to thank Dr. S. Gurusiddaiah, Associate Director, Bioanalytical Centre, Washington State University, Pullman, Washington, USA for helping in some bio-analytical experiments. The help rendered by Dr. D. Rajagopal Rao, Biochemistry Division, Central Food Technological Research Institute, Mysore is gratefully acknowledged. We wish to acknowledge the gift of some inhibitors used in this study by Pesticides and Industrial Chemical Repository, MD-8, Research Triangle Park, North Carolina, USA and CIBA-GEIGY Ltd., Basel, Switzerland. We also thank Dr. G. K. N. Reddy and Dr. T. K. Virupaksha, for their helpful suggestions.

References

Albersheim, P., Nevins, O. J., English, P. D. and Karr, A. (1967) *Carbohydr. Res.,* **5,** 340.
Aldridge, W. N. (1950) *Biochem. J.,* **46,** 451.
Aldridge, W. N. and Reiner, E. (1972) *Enzyme Inhibitors as Substrates* (Amsterdam: North Holland Publishing Company).
Andrews, P. (1964) *Biochem. J.,* **91,** 222.
Augustinsson, K. B. (1948) *Acta Physiol. Scand.,* **15,** 1.
Axenfors, B., Anderson, I. and Augustinsson, K. B. (1979) *Biochim. Biophys. Acta.,* **570,** 74.
Barker, D. L. and Jenks, W. P. (1969) *Biochemistry,* **8,** 3890.
Bartley, I. M. and Stevens, W. H. (1581) *J. Exp. Bot.,* **32,** 741.
Bencze, W. L. and Schmid, K. (1957) *Anal. Chem.,* **29,** 1193.
Bhatia, C. R. and Nelson, J. P. (1969) *Biochem. Genet.,* **3,** 207.
Bjorndal, H., Lindberg, B. and Svensson, S. (1967) *Carbohydr. Res.,* **5,** 433.
Carino, L. A. and Montgomery, M. W. (1968) *Phytochemistry,* **7,** 1483.
Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.,* **121,** 404.
Desborough, S. and Peloquin, S. J. (1968) *Am. Potato J.,* **45,** 220.
Earnst, M. and Hartmann, E. (1980) *Plant Physiol.,* **65,** 447.
Ellman, G. L. (1959) *Arch. Biochem. Biophys.,* **82,** 70.
Ellman, G. L., Courtney, K. D., Andres, Jr. V. and Featherstone, R. M. (1961) *Biochem. Pharmacol.,* **7,** 688.
Gomori, G. (1953) *Lab. Clin. Med.,* **42,** 445.
Hofstee, B. H. J. (1960) *Enzymes,* **4,** 485.
Holmes, R. S. and Masters, C. J. (1967) *Biochim. Biophys. Acta,* **132,** 379.
Hunter, R. L. and Markert, C..L. (1957) *Science,* **125,** 1294.
Iwai, M., Okumara, S., Delear, E. L. and Tsujisaka, Y. (1983) *Agric. Biol. Chem.,* **47,** 1865.
Karnovsky, M. J. and Roots, L. (1964) *J. Histochem. Cytochem.,* **12,** 215.

Kasturi, R. and Vasantharajan, V. N. (1976) *Phytochemistry,* **15,** 1345.
Krisch, K. (1972) *Enzymes,* **5,** 43.
Lambardo, D., Guy, O. and Figarella, C. (1978) *Biochim. Biophys. Acta,* **527,** 142.
Levy, M. and Ocken, P. R. (1969) *Arch. Biochem. Biophys.,* **135,** 259.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.,* **193,** 265.
Macko, V., Honald, G. R. and Stahmann, M. A. (1967) *Phytochemistry,* **6,** 465.
Matsunaga, A., Koyama, N. and Nosoh, Y. (1974) *Arch. Biochem. Biophys.,* **160,** 504.
Mckelvy, J. F. and Lee, Y. C. (1969) *Arch. Biochem. Biophys.,* **132,** 99.
Montgomery, M. W., Norgaard, M. J. and Veerabhadrappa, P. S. (1968) *Biochim. Biophys. Acta.,* **167,** 567.
Moore, S and Stein, W. H. (1963) *Methods Enzymol.,* **6,** 819.
O'Brien, R. D. (1960) *Toxic Phosphorus Esters* (New York: Academic Press) p. 76.
Okumara, S., Iwai, M. and Tsujisaka, Y. (1983) *Agric. Biol. Chem.,* **47,** 1869.
Oosterbaan, R. A. and Jansz, H. S. (1965) in *Comprehensive Biochemistry: Cholinesterases, esterases, and lipases* (eds M. Florkin and E. H. Stotz) (Amsterdam: Elsevier) vol. 16, p. 1.
Ornstein, L (1964) *Ann. N.Y. Acad. Sci.,* **121,** 321.
Payne, R. C. and Koszykowski (1978) *Crop Sci.,* **18,** 557.
Premaratna, A., Shadaksharaswamy, M. and Nanjappa, S. (1981) *Indian J. Biochem. Biophys.,* **18,** 32.
Rennert, O. M. (1967) *Nature* (*London*), **213,** 1133.
Sae, S. W., Kadoum, A. M. and Cunningham. B. A. (1971) *Phytochemistry,* **10,** 1.
Scott, K. and Zerner, B. (1975) *Can. J. Biochem.,* **53,** 561.
Upadhya, G. A., Govardhan, L. and Veerabhadrappa, P. S. (1985) *J. Biosci.,* **7,** 289.
Van Asperen, K. (1962) *J. Insect. Physiol.,* **8,** 401.
Veerabhadrappa, P. S., Marcus, S. R. and Shadaksharaswamy, M. (1980) *Indian J. Biochem. Biophys.,* **17,** 186.
Veerabhadrappa, P. S. and Montgomery, M. W. (1971) *Phytochemistry,* **10,** 1175.
Veerabhadrappa, P. S. and Upadhya, G. A. (1979) *Indian J.Exp. Biol.,* **17,** 640.
Veeraswamy, M., Nagaraja, M. S., Veerabasappa Gowda, T., Seshadri, H. S. and Ramaiah, T. R. (1973-74) *J. Mysore University* (*Section B*), **26,** 156.
Weber, K. and Osborn, N. (1969) *J. Biol. Chem.,* **244,** 4406.
Wringley, C. W. (1969) *Shandon Instrument Applications,* No. 29,1.