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Properties of Carboxylesterase (E.C. 3.1.1.1) from *Brithys crini* (F.) (Lepidoptera: Noctuidae)

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The carboxylesterase (E.C.3.1.1.1) obtained from *Brithys crini* (F) hydrolyzed *ortho*-nitrophenyl acetate (*o*-NPA) and thiophenyl acetate (TPA) and this followed normal Michaelis kinetics at low substrate concentrations, but deviated significantly from it at high substrate concentrations. The enzyme showed activation at high substrate concentration with *o*-NPA as substrate and inhibition with TPA as substrate. An interacting catalytic site was present beside, the esteratic site on the enzyme.

Key Words: **Carboxylesterase, Substrate activation, Substrate inhibition,** *Brithys crini*

Introduction

Carboxylesterases (E.C. 3.1.1.1) (CE) are ubiquitous and have been implicated in the metabolism of diverse groups of compounds (Krisch 1971, Ecobicon 1979). In insects, especially, their importance in the metabolism of lipids (Stevenson 1969), xenobiotics (Mehrotra & Singh 1976), insecticides—organophosphates, carbamates and pyrethrins (Ecobicon 1979, Plapp 1976, Motoyama et al. 1980) and in the regulation of juvenile hormone titres (de Kort & Granger 1981) has been emphasized. The enzyme has been demonstrated occurring in a number of insect species (Houk & Hardy 1981). However, the kinetic properties of the enzyme obtained from various insect sources may differ significantly, as is true for its mammalian counterpart (Phokela et al. 1981). The present study reports the kinetic parameters of CE obtained from *Brithys crini* (F.),—a pest of lily plant.

Materials and Methods

The fifth instar larvae of *Brithys crini* were gathered from the lily plants grown at the campus of the Indian Agricultural Research Institute, New Delhi. Acetone powders of the larvae were prepared according to Mehrotra (1962) by homogenizing larvae in cold acetone. The

volume of acetone used for making acetone powders was 15 times the weight of the larvae. The CE was extracted from the acetone powders in phosphate buffer (*p*H 8.0, 0.1M), and was partially purified by freezing and thawing twice. This treatment led to the precipitation of large amounts of extraneous proteins which were eliminated by centrifugation. The extract was divided into small aliquotes and kept at —5°C until used.

The CE activity in the extracts was assayed using two different substrates, *ortho*-nitrophenyl acetate (*o*–NPA) and thiophenyl acetate (TPA) according to Phokela et al. (1981). The reaction rates were calculated by using the molar extinction coefficients of 4.95×10^3 for *o*–NPA and 1.36×10^4 for TPA. The kinetic parameters of the enzyme were determined by estimating the initial rate of reaction at various concentrations of the substrates. Eighteen concentrations of *o*–NPA ranging from 0.25 mM to 5.0 mM and 17 concentrations of TPA ranging from 0.5 mM to 5.0 mM were used. All esterase assays using either *o*–NPA or TPA were carried out at 30°±1°C in phosphate buffer (*p*H 8.0, 0.1M). The reactions were followed by observing the change in optical density at 415 nM using an ECIL spectrophotometer Model GS 865. Proteins were estimated spectrophotometrically at 280 nM according to Dixon and Webb (1964). Calculations of kinetic parameters were done using a programable calculator (Hindustan Micro–2200) with extended memory. The programme*, simultaneously calculated K_m (Michaelis constant), V_{max} (maximal velocity and k (first order reaction constant) by three linear transformations of Michaelis-Menten equation (Lineweaver & Burk 1934, Hanes 1932, Hofstee 1952) and the statistical method of Wilkinson (1961).

Chemicals

TPA was prepared in the laboratory (Augustinsson et al. 1972) while the *o*–NPA was obtained from Eastman Kodak Co. Rochester. 5,5–dithiobis–(2–nitrobenzoic acid) (DTNB) was the product of Sigma Chemical Co. Ltd., St. Louis, Mo. Other chemicals used were analytical grade reagents of the highest purity.

Results

The CE obtained from *Brithys* hydrolyzed both *o*–NPA and TPA as has been also reported for mammalian enzyme (Krisch 1966) and the enzyme obtained from insects *Euproctis lunata* (Mehrotra & Singh 1976), *Aphis craccivora* and *Lipaphis erysimi* (Phokela et al. 1981).

(a) *Hydrolysis of o–NPA*

Data on hydrolysis of various concentrations of *o*–NPA by the enzyme plotted according to Lineweaver and Burk (1934) are presented in figure 1A. From the data presented it would be seen that the enzyme followed the normal Michaelis kinetics within a limited range (0.25 mM to 2.0 mM) of low substrate concentrations and deviated from it at high substrate concentrations. Kinetic parameters of *o*–NPA hydrolysis, in the range in which it followed the normal Michaelis kinetics calculated by the methods of Lineweaver and Burk (1934), Hanes (1932), Hofstee (1952) and the statistical method of Wilkinson (1961) are presented in table 1. At high substrate concentrations (2.25 mM — 5.0 mM) the enzyme showed activation. From the results presented it will be seen that at

*The programme is available on request.

higher substrate concentrations the K_m increased nearly 7–fold, the V_{max} 2.5–fold but the k value (V_{max}/K_m) decreased by half from the corresponding values at low substrate concentrations. Activation of *o*–NPA hydrolysis activity of CE has also been observed previously for the enzyme obtained from mammalian sources (Greenzaid & Jencks 1971, Levy & Ocken 1969) and aphids (Phokela et al. 1981).

(b) *Hydrolysis of TPA*

Hydrolysis of TPA by the CE also deviated from the normal Michaelis kinetics at high substrate concentrations (figure 1B) but behaved normally at lower substrate concentrations. The kinetic parameters calculated for low substrate concentrations (0.5 — 2.25 mM) are presented in table 2. At high substrate concentrations (2.5 mM — 5.0 mM) the enzyme showed substrate inhibition. A Dixon and Webb (1964) plot also showed that the enzyme was inhibited at high substrate concentration with the k (dissociation constant of the inactive enzyme-substrate complex) being 11.4 mM, The substrate inhibition of the enzyme using this substrate has also been observed for pig liver esterase (Greenzaid & Jencks 1971).

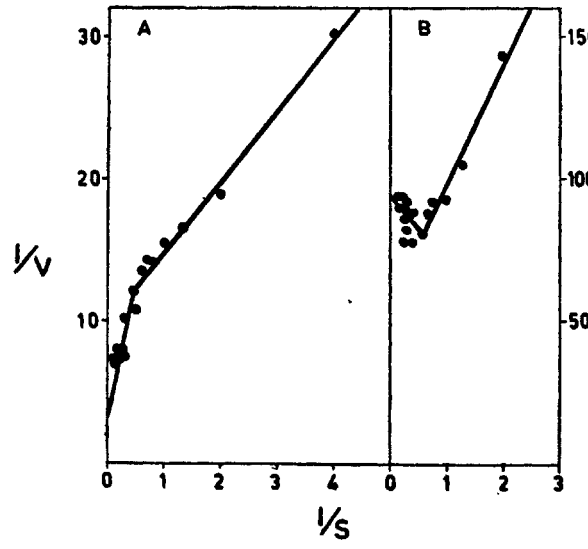

Figure 1 Lineweaver and Burk plots of carboxylesterase activity using (A) *o*-NPA and (B)TPA as substrate. (The composition of the reaction mixture was the same as given under table 1 for *o*-NPA and table 2 for TPA)

Discussion

A comparison of the kinetic parameter for the hydrolysis of the two substrates showed that the K_m of CE for *o*–NPA and TPA did not differ significantly suggesting that the affinity of the enzyme for both the substrates was similar. The V_{max} value of the enzyme was, however, 10 times higher in case of *o*–NPA than TPA. This fact was further emphasized by the differences in k values which were 7–fold higher for o–NPA than TPA. Another point of difference between the two substrates was that the enzyme showed substrate activation at higher substrate concentrations (above 2.0 mM) in case of *o*–NPA whereas in case of TPA the enzyme showed substrate inhibition (above 2.25 mM). Although substrate activation of CE activity has been observed in a number of preparations obtained from various sources, in this respect enzyme from *Brithys* is similar to that from aphids (Phokela et al. 1981) and other mammalian sources (Greenzaid & Jencks 1971, Levy & Ocken 1969). The substrate-inhibition, using TPA, has been reported only from pig liver CE (Greenzaid & Jencks 1971).

Deviations from the normal Michaelis kinetics, specially activation at high substrate concentrations have been observed in CEs obtained from various sources (Greenzaid & Jencks 1971, Levy & Ocken

Table 1 *Kinetic parameters of the carboxylesterase obtained from* Brithys crini *larvae using o-NPA as substrate*

Substrate range	K_m (mM)	V_{max} mM/lt/min/ mg protein	k/min	r	Method
0.25mM–2.0mM	0.5443	0.1040	0.1911		Wilkinson (1961)
(8 observations)	0.4975	0.1006	0.2022	0.9888**	Lineweaver & Burk (1934)
	0.5794	0.1059	0.1827	0.9709**	Hanes (1932)
	0.4842	0.1000	0.2065	0.9137**	Hofstee (1952)
Mean ± Standard	0.5264±	0.1026±	0.1956±		
deviation	0.0438	0.0028	0.0108		
2.25mM–5.0mM	3.0477	0.2280	0.0748		Wilkinson (1961)
(10 observations)	5.7435	0.3114	0.0542	0.8754**	Lineweaver & Burk (1934)
	3.4118	0.2348	0.0688	0.8119**	Hanes (1932)
	0.8109	0.1504	0.1854	0.2101N.S.	Hofstee (1952)
Mean ± Standard	4.0677±	0.2581 ±	0.0659±		
deviation	1.4627	0.0463	0.0106		

Note : The mean of K_m, V_{max} and k at high substrate concentrations were calculated by excluding the method of Hofstee (1952) as it gave very low value of r

**Significant at 1% level

The incubation mixture, in addition to 4 mg protein contained the following: (μ moles); Varying concentration of *o*–NPA in 0.1 ml of CH_3OH; phosphate buffer (*p*H 8.0), 200; in a total volume of 2.0 ml

Table 2 *Kinetic parameters of the carboxylesterase obtained from* Brithys crini *larvae using TPA as substrate*

Substrate range	K_m (mM)	V_{max} (mM/lt/mg protein/min)	k/min	r	Method
	0.5780	0.0162	0.0280		Wilkinson (1961)
	0.6818	0.0171	0.0251	0.9815**	Lineweaver & Burk (1934)
0.5 mM 2.25 mM	0.5729	0.0161	0.0281	0.9955**	Hanes (1932)
(7 observations)	0.5912	0.0163	0.0275	0.9428**	Hofstee (1952)
Mean ± Standard	0.6060±	0.0164±	0.0272±		
deviation	0.0511	0.0005	0.0014		

** Significant at 1% level

The incubation mixture, in addition to 1.35 mg protein contained the following (μ moles): Varying concentrations of TPA in 0.1 ml CH_3OH; DTNB, 0.8, phosphate buffer (*p*H 8.0), 200; in a total volume of 2.0 ml

1969, Adler & Kistiakowsky 1962, Barker & Jencks 1969). A number of possibilities have been discussed in this regard. It is most likely that the activation of *o*-NPA was due to the presence of two interacting catalytic sites besides the primary esteratic site. It is likely that the presence of electrophilic inductive substituent NO_2 in *o*-NPA would induce a partial positive change on the alcoholic oxygen of the ester and consequently help in the faster hydrolysis of substrate. The absence of any electrophilic group on TPA, naturally, will slow down the rate of reaction. How this inhibition in esterase activity is brought about at excess TPA concentration is not as yet fully understood and needs further investigations. Since normal Michaelis kinetics have also been reported from CEs obtained from both insects (Mehrotra & Singh 1976, Phokela et al. 1981) and mammals (Barker & Jencks 1969, Stoops et al. 1969), it suggests that the enzymes obtained from various sources differ significantly in their catalytic properties from each other. CEs are a family of enzymes which show interspecific difference (Stoops et al. 1969). These differences may have great physiological significance and may explain the profile of xenobiotic metabolism and resistance or susceptibility to insecticides.

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