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KINETIC STUDIES ON CARBOXYLESTERASE OF MODEL NEMATODE *Caenorhabditis elegans* EXPOSED *in vitro* TO DICHLORVOS

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ABSTRACT

The study was aimed to understand the kinetics of organophosphorus detoxifying enzyme carboxylesterase (EC 3.1.1.1) and to know the its sensitivity in *Caenorhabditis elegans*, a moist soil dwelling nematode used as model system for studying xenobiotics. Enzymatic hydrolysis of ρ-nitrophenylacetate (PNPA) showed a K_m value of 20.95 µM, V_{max} of 4.62 nmol min^{-1} mg^{-1} protein and substrate saturation of 350 µM for carboxylesterase (*CaE*) *in vitro* kinetics on exposure to dichlorvos from *C. elegans*. The IC_{50} with dichlorvos, an organophosphorus compound, was 81.16 nm at excess of K_m. LB plot showed the K_m, V_{max} and V_{max}/K_m values of inhibited CaE: 19.57 µM, 2.88 nmol min^{-1} mg^{-1} protein and 0.14 min^{-1} mg^{-1} protein, respectively, at IC_{50} concentration of dichlorvos. Inverse LB plot revealed the inhibition of CaE in *C. elegans* irreversible in presence of dichlorvos. Hence this may be used as invertebrate model to understand the other targets on exposure to dichlorovos.

Key words: *Caenorhabditis elegans*, carboxylesterase, kinetics, dichlorvos and Lineweaver-Burk plot.

INTRODUCTION

Organophosphorus compounds (OPs) are widely used as pesticides and chemical warfare agents (Zheng *et al.*, 2013). These synthetic compounds get deposited into the environment and cause serious pollution problems. These insecticides exert main toxicological effects through irreversible phosphorylation of esterases in central and peripheral nervous system (Aldridge and Reiner, 1972). Carboxylesterases (CaE), EC 3.1.1.1, are a class of esterases that hydrolyze ester-containing compounds into their corresponding alcohol and acid (Wheelock *et al*., 2005). CaE are involved in detoxification of OP compound via scavenging mechanism which has been considered as an efficient way of detoxification (Prabhakaran and Kamble, 1996).

Chlorpyrifos reportedly inhibited carboxylesterase in Chinook Salmon at concentrations that failed to inhibit AChE. The greater affinity of enzyme for OP compounds has earned them the reputation of sink for OP compounds, which has been proposed as a protective mechanism for OP toxicity (Maxwell *et al.*, 2008). Dichlorvos exerts toxic effects by inhibiting esterases, especially AChE. In addition, the production of different forms of carboxylesterases reportedly causes dichlorvos toxicity and resistance in several insect species (Wang *et al*., 2004).

Large quantities of chemical insecticides are applied in fields and indoors every year which directly or indirectly add to the selection pressure on vector mosquitoes. *Culex pipiens* complex has evolved to be resistant to all types of chemical insecticides, especially to organophosphates, through carboxylesterases. Six resistant carboxylesterase alleles (ester) were recorded in China previously and sometimes co-existed. The worms exposed to dichlorvos showed time-dependent increase in the expression of genes involved in stress responses, and predominant effect was observed on metabolic processes, while at later times an immune-like response and cellular repair mechanisms were observed (Lewis *et al*., 2009). Carboxylesterases are more sensitive to inhibition by OPI than AChE (Wheelock *et al.,* 2005). The relevance of carboxylesterase in toxicity of OPI is surprising that the enzyme as a target for inhibition has received less attention. Carboxylesterase, particularly the β-esterses, are known to be inhibited by OP compounds, a property of the enzyme which has given the status of detoxification mechanism to the enzyme with regard to OPI toxicity.

Carboxylesterases are the main detoxification enzymes which play an important role in insecticide resistance and have been associated with resistance to several insecticide classes in many insects. Due to its role in insecticide metabolism, the measurement of carboxylesterase activity is used as a biochemical indicator of insecticide resistance in many insect species (Silas *et al*., 2008). OPs and carbamates have increased affinity to carboxylesterase over acetylcholine-esterase. Currently researchers have focused on the use carboxylesterase activity as a biomarker of organism exposure or susceptibility to agrochemicals. Very limited work has been conducted in this area and those too are focused on esterase levels in fish (James *et al.*, 1986; Wogram *et al.*, 2001). Carboxylesterase activity in relation to agrochemical exposure has been examined in additional species and may prove useful in ecosystem-wide environmental monitoring.

The nematode *Caenorhabditis elegans* is used in various studies on neurobiology, genetics, developmental biology apoptosis and toxicity because of its well-characterized genome, ease in maintenance, short and prolific life cycle and small body size. These attributes have led to increased use of *C. elegans* in toxicology, both for mechanistic studies and high-throughput screening (Maxwell *et al*., 2008). Our earlier studies have demonstrated the inhibitory effects of dichlorvos on AChE in *C. elegans in vivo* (Rajini *et al*., 2008; Jadhav and Rajini, 2009 a,b). Similarly, rat brain homogenate system has extensively been used *in vitro* to understand enzyme inhibition patterns by OP's (Jadhav *et al*., 2014). Reports have revealed the expression of heat shock proteins, decreased brood size, paralysis and nose contraction on exposure to dichlorovos (Jadhav and Rajini, 2009 a,b). Hence, it was of interest to investigate the use of *C. elegans* homogenate system in determining the basic kinetic parameters of carboxylesterase and establish the nature of inhibition by dichlorvos.

MATERIALS AND METHODS

Bovine serum albumin [BSA] (fraction V) and ρ-nitrophenylacetate (PNPA) were procured from Sigma Chemicals (USA) and Sisco Research Lab. (Mumbai, India), respectively. Dichlorvos (technical grade; 97.6%) was gifted by Hyderabad Chemical Supplies Ltd. (India). The other reagents used were of analytical grade. Stock solution (5 mM) of dichlorvos (DDVP) was prepared in double distilled water and diluted to obtain working solutions for *in vitro* assays. All the experiments were repeated three times.

Worm culture and preparation of C. elegans homogenate

The nematodes were cultivated on nematode growth medium (NGM) comprising of 3 g $L^{-1,}$ NaCl, 2.5 g L^{-1} proteose peptone, 5 mg L^{-1} cholesterol, 1 mMol L^{-1} $CaCl_2$, 1 mol L^{-1} $MgSO_4$, 25 mmol L^{-1} potassium phosphate, and 17 g L^{-1} agar (pH 6.0) on an established lawn of *Escherichia coli* strain OP-50. and maintained at 20°C. The worms used for the assays were larvae L4 (3.5 d) obtained after a synchronous culture. The worms were prepared for the assays by washing the worms from the petriplates into a centrifuge tube and allowing them to settle by gravity. The worms were rinsed

three times with K-medium and then used for the *in-vitro* assay conditions. Worm (gravid stage/3.5 d old) were harvested and homogenized in 50 mM tris-HCl (pH 7.4). The homogenates were centrifuged at 10,000 rpm for 10 min. and supernatant stored at –20°C until assay.

Substrate saturation

The homogenate of *C elegans* was used as enzyme source to determine the kinetic parameters for carboxylesterase (CaE) activity. Carboxylesterase was assayed with the substrate, ρ-nitrophenyl-acetate (PNPA) by the method of Sidhu and Blair (1975). Enzyme sample (50 µL) was added to 3 mL tris-HCI buffer (50 mM; pH 7.4) and pre-incubated for 10 min. at 30ºC in a water bath. K_m and V_{max} of substrate PNPA were determined by assaying the activity with increasing substrate concentration (2.5 - 500 µM dissolved in acetone). The reaction mixture was incubated for 30 min. at 30°C. The absorbance of reaction product ρ-nitrophenol was measured at 405 nm against a reagent blank containing PNPA and buffer in a UV-VIS spectrophotometer. Enzyme activity (CaE-PNPA) was calculated using the extinction co-efficient for ρ-nitrophenol (€405 = 1.83 x 10^4 M^{-1} cm^{-1}). The rate (V) was plotted against the substrate concentrations by inverse Lineweaver and Burk plot (Lineweaver and Burk, 1934) to obtain Michaelis constant (K_m), maximum velocity (V_{max}) and catalytic efficiency (V_{max}/K_m).

Determination of IC_{50}

The *C. elegans* carboxylesterase enzyme (50 µL) in 50 mM tris-HCI buffer (pH.7.4) was incubated with different concentrations of dichlorvos ranging from 10 - 200 nM at 30°C for 30 min. The residual *CaE* activity was assayed with increasing substrate concentration (2.5 - 500 µM dissolved in acetone), as described earlier. The median inhibitory concentration IC_{50} was determined by linear regression of inhibition curve (Finney, 1971).

Kinetic properties of carboxyl esterase (CaE) by dichlorvos

To determine the nature of inhibition of CaE activity *in vitro* by dichlorvos, the enzyme was incubated with IC_{25}, IC_{50} and IC_{75} concentrations of dichlorvos in 3.0 mL tris-HCI buffer for 30 min. at 30ºC. The residual activity for each inhibitor concentration was determined with increasing substrate (PNPA) concentrations. The K_m, V_{max} and nature of inhibition was determined from the Lineweaver-Burk plot (1/V against 1/S).

Protein estimation

For protein estimation the method of Lowry *et al.* (1951) was followed, wherein 10 µL *C. elegans* homogenate was added to 490 µL distilled water and incubated with 2.5 mL alkaline copper sulfate for 10 min. at room temperature. Then 250 µL Folio's reagent (1:1 diluted with water) was added and colour read after 30 min. at 670 nm. BSA was used as standard.

RESULTS AND DISCUSSION

Carboxylesterase from *C. elegans* homogenate system hydrolyzed the substrate PNPA in a typical Michaelis-Menten pattern with the hydrolysis rate directly proportional to the substrate concentrations in initial phase followed by the attainment of zero order kinetics with respect to the substrate concentrations. Interestingly, higher concentrations of PNPA (>350 µM) inhibited the enzyme activity. The double reciprocal LB-plot revealed K_m of enzyme for substrate hydrolysis as 20.95 µM while maximum velocity attained (V_{max}) 4.62 nmol min^{-1} mg^{-1} protein (Fig. 1 and 2). Similarly, in striped-flea-beetle (*Phyllotreta striolat*) the populations were exposed to dichlorvos twice consecutively to obtain high resistance individuals. The Insecticide suscepti-bility, activities of carboxyl-esterase (CarE), acetylcholine esterase (AChE) and glutathione-S-transferase (GST), and

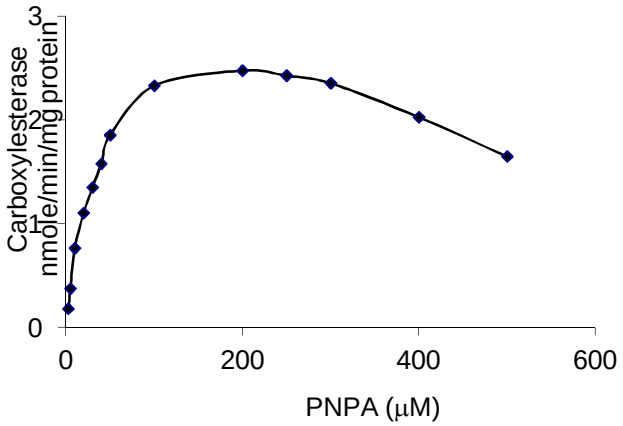

Fig. 1: Effect of substrate (PNPA) concentrations on carboxylesterase activity in *C. elegan*

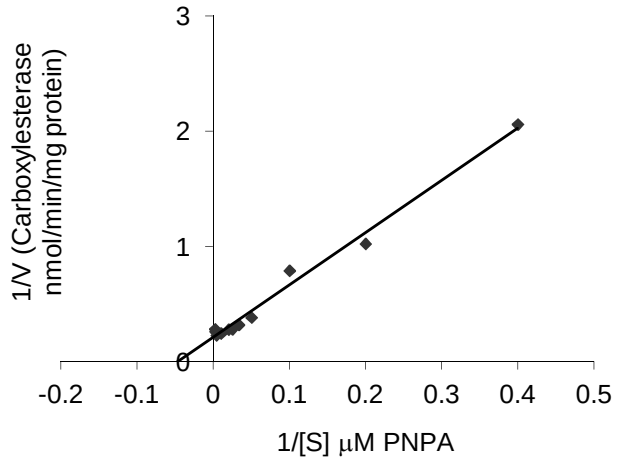

Fig. 2: Lineweaver-Burk plot for carboxylesterase activity in *C. elegans*

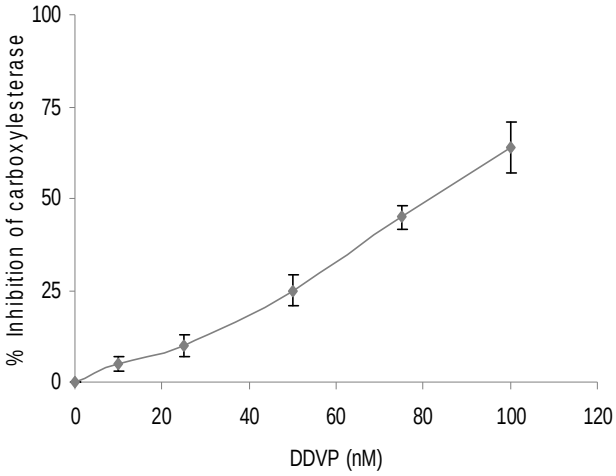

Fig. 3: Inhibition pattern of carboxylesterase by dichlorvos (DDVP) in *C. elegans* homogenate

kinetic constants were determined. The results suggested that the LC_{50} value of SP was slightly higher than CP. The maximum velocity (V_{max}) and Michaelis constant (K_m) of CaE in SP were much less than that in CP. The AchE activity of SP was 0.0074 OD_{412}·$insect^{-1}$·min^{-1}, significantly lower than that of CP (0.0096 OD_{412} $insect^{-1}$ min^{-1}). Their study also suggested that the resistance of SFB to dichlorvos might relate to the decreased activity of AChE as well as the increased activities of CaE and GST (Li-zhen *et al*., 2009). The potential of dichlorvos for organophosphate interactions, dose- and time-related inhibition of cholinesterase and carboxylesterase activities were studied in male mice and they observed the inhibition of cholinesterase and carboxyl esterase activities (Cohen and Marion, 1976). Lewis *et al*. (2009) have suggests that an insecticide can elicit multiple ester alleles and one ester allele can work on multiple insecticides. The evolutionary scenario of carboxylesterases under insecticide selection is possibly "one to many". The IC_{50} for carboxylesterase of *C. elegans* was 81.16 nM at excess K_m concentration of the substrate, ρ-nitro-phenyl acetate. The LB plot, K_m, V_{max} and V_{max}/K_m values of inhibited CaE were 19.57 µM, 2.88 n mol min^{-1} mg^{-1} protein and 0.14 min mg^{-1} protein, respectively (Fig. 3, Table 1) at IC_{50} of dichlorvos. The *CaE* expression was upregulated in *Aphis gossypii* adults of both dimeth-oate- and malathion-resistant strains, showing the potential involvement of *CaE* in organophosphorus insecticide resistance. Functional analysis (RNAi) was hence carried out which suggested that CaE is involved in OP resistance development (Gong *et al*., 2014).

Dichlorvos inhibited the enzyme activity *in vitro* in concentration dependent manner with regression analysis showing IC_{50} of dichlorvos against carboxyl-esterase activity as 81.16 nm. The double reciprocal LB-plot revealed a similar inhibition pattern for carboxyl esterase by dichlorvos as compared to the inhibition of AChE

Table 1: Kinetic charecteristics of *C. elegans* CaE in the presence of dichlorvos

Dichlorvos (nM)	K_m (μM)	V_{max} (nmol/ min/ mg protein)	V_{max}/K_m
IC_{25}	18.69	3.45	0.18
IC_{50}	19.57	2.88	0.14
IC_{75}	17.58	2.28	0.12

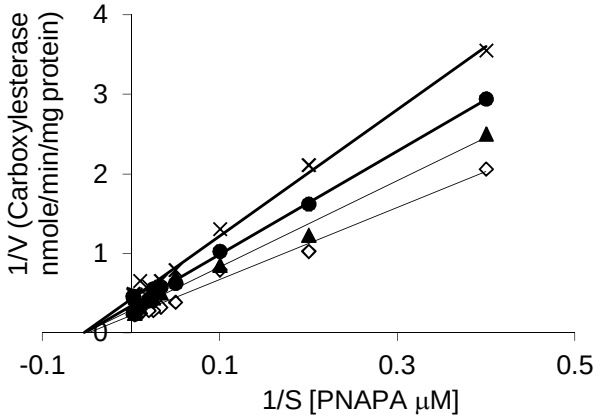

Fig. 4: Inhibition of *C. elegans* CaE activity with IC_{25}, IC_{50} and IC_{75} concentrations of dichlorvos

reported previously. *In vitro* exposure of *C. elegans* homogenate system to various concentrations (IC_{25}, IC_{50} and IC_{75}) of dichlorvos resulted in alterations in enzymes kinetic parameters typical of irreversible inhibition. The V_{max} of inhibited enzyme progressively decreased while K_m values remained unchanged. As a consequence, the catalytic efficiency (V_{max}/K_m) decreased with increase in dichlorvos concentration.

Exposure of C. *elegans* for 8 hr to OP treatment altered the expression of 87 genes. The abundance of 34 proteins also changed in OP-exposed worms. Many genes and proteins affected by OPs are expressed in neuronal and muscle tissues and have been found involved inlipid metabolism, cell adhesion, apoptosis/ cell death and detoxification. Twenty-two genes were affected by two cytochrome P450s, UDP-glucuronosyl/ UDP-glucosyl-transferases, or ρ-glycoproteins. The OPs; a large amount of these encode abundance of transcripts and the proteins they encode were well correlated.

In serum and liver of mammals a number of β-esterases exist with no known functions. These enzymes are considered to be involved in detoxification of OPI and carbamates, as each molecule of these enzymes can scavenge a minimum of one insecticide molecule before it reaches its target in nervous system (Sogorb *et al.,* 2002). The gene expression studies in *C. elegans* by two OPs evoked changes in gene expression and half of them were involved in detoxification (Lewis *et al.,* 2013).

The nematode *C. elegans* under natural conditions is inhabitant of soil ecosystem, particularly where moist micro-environments exists (Boyd *et al.,* 2003). These micro-environments are at high risk of contamination with the run-off agrochemicals particularly insecticides. The present work clearly demonstrates that dichlorvos inhibited carboxylesterase of *C. elegans* by irreversible mechanisms. It is extremely important to note that carboxylesterase operates as a target for OPI. The presence of such target machinery could serve as a detoxification mechanism that may protect the worms from OPI toxicity and thereby help the survival of nematodes.

Conclusion: The study revealed that the substrate PNPA has saturation at 350 μM and inverse of LB plot showed CaE inhibition in *C. elegans* irreversible in nature in presence of dichlorvos. The enzyme inhibition may be used in environmental monitoring of OP contaminants in run-off water.

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