In vitro Effects of Chlorpyrifos on the Acetylcholinesterase Activity of Euryhaline Fish, *Oreochromis mossambicus*

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The *in vitro* effect of a widely used organophosphorus insecticide, chlorpyrifos (CPP), on the acetylcholinesterase (AChE) activity was studied *in vitro*. The kinetic constants K_m and V_{max} and the bimolecular constant k_i were determined *in vitro*. The *in vitro* AChE study indicated that CPP is neurotoxic and that it alters the apparent K_m values widely in a concentration-dependent manner, resulting in a competitive type of inhibition. Based on the k_i values, the sensitivity of AChE in brain is greater than that in gill tissue, at $7.3 \cdot 10^{-5}$ M and $11.92 \cdot 10^{-5}$ M, respectively. The study points to the importance of kinetic studies and the results suggest that in biomonitoring programmes brain AChE activity can be a good diagnostic tool for CPP toxicity.

Key words: Acetylcholinesterase, Chlorpyrifos, *Oreochromis mossambicus*

Introduction

The activity of cholinesterase in fish is being used as a biomarker for aquatic pollution. *Tilapia* sp. was used for biomonitoring of organophosphorus (OP) pollutants in the aquatic environment. It is a widespread food fish species in tropical environments especially in Asia, South America, and Africa and is commonly found in brackish water in estuaries around the world (Vijayan *et al.*, 1996). The measurement of the acetylcholinesterase (AChE, E.C. 3.1.1.7) inhibition has been widely used in monitoring the exposure of OP and carbamate pesticides (Dembele *et al.*, 2000; Fulton and Key, 2001).

In vitro kinetic constants of AChE against a specific toxicant provide reference values, which can be used as a promising tool for environmental screening and monitoring. *In vitro* systems have been suggested as economical and efficient alternatives for animal testing for OP toxicity (Barber *et al.*, 1999). The aim of the present study was to investigate the sensitivity of the AChE activity of euryhaline fish (*Oreochromis mossambicus*) brain and gill against chlorpyrifos [CPP, *O*,*O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate] *in vitro* and to determine the kinetic parameters of this enzyme.

Material and Methods

Test chemicals

All reagents used were of analytical grade and were used without any further purification. Acetylthiocholine iodide (ATC) and 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The technical grade insecticide CPP used in the experiment was of 99.9% purity. It was received free from Hyderabad Chemicals Products Ltd. (Industrial Estate, Balanagar, Hyderabad, Andhra Pradesh, India).

Test organisms

Euryhaline fish (*Oreochromis mossambicus*) were collected from Kapra Lake (Hyderabad, India), which is relatively free of pollutants, and were brought to the laboratory in large aerated drums. Later, they were acclimatized for two weeks in a huge cement tank ($8 \times 6 \times 4$ m) and fed with commercial dry feed pellets (Hello Fish-Dry pellets, CVM Products & Co., Beijing, China). The natural photoperiod of 13 h light/11 h dark was maintained.

Tissue preparation

Fish weighing (5 ± 1) g were anesthetized by placing them for 10 min in benzocaine hydrochloride (200 mg l^{-1}), and tissues like brain and gill were excised. Tissues were homogenized (10% w/v) in 0.1 M phosphate buffer (pH 7.4) containing Triton-X 100 and 1 mM EDTA, using a Potter-Elvehjam homogenizer fitted with a Teflon pestle. The homogenates were centrifuged at $500 \times g$ for 10 min, and the supernatant was further centrifuged at $12000 \times g$ for 20 min. The resultant supernatant (post-mitochondrial supernatant) was used as the enzyme source for estimation of the AChE activity. Enzyme preparation was carried out at 4 °C. Protein was estimated by the Brad-

ford method (Bradford, 1976) using bovine serum albumin as standard.

AChE activity

The AChE activity was determined in brain and gill tissues by the method described by Ellman *et al.* (1961). A typical run for all experiments in 96-well plates consisted of 75 μl of 0.1 M phosphate buffer, pH 7.5, 25 μl of DTNB (0.16 mM), 25 μl of ATC, and 25 μl of protein (0.1 mg) for each well. CPP at various concentrations ($3.56 \cdot 10^{-5}$, $7.13 \cdot 10^{-5}$, $10.69 \cdot 10^{-5}$, and $14.26 \cdot 10^{-5}$ M) prepared in acetone along with various substrate concentrations (0.04, 0.05, 0.08, 0.1, 0.2, and 0.4 mM) were added simultaneously to react with the enzyme. The reaction was initiated by adding the substrate at 27 °C, and the colour development was recorded continuously for 5 min at 412 nm in a spectrophotometer using SoftMax Pro 5. The AChE activity was calculated as μmol of acetylthiocholine hydrolyzed min^{-1} mg $protein^{-1}$.

The kinetic parameters K_m and V_{max} were determined by computer analyses of Lineweaver-Burk or double reciprocal plots of experimental data using five concentrations of substrate, ranging from 0.04 to 0.2 mM.

Statistical analysis

All values presented are means ± SE (standard error). The experiments were repeated on three different occasions in triplicate and the data were analyzed by Student *t*-test. $P < 0.05$ was accepted as statistically significant.

Results and Discussion

The sensitivity of AChE inhibition *in vitro* appears to be a principle determinant to compare the toxicity of molecules. The biochemical enzymatic variations are powerful predictive tools in the assessment of toxicity (Rahman *et al.*, 1999). Double reciprocal plots of the initial velocity *versus* substrate concentration for brain and gill AChE in the presence of various CPP concentrations are presented in Fig. 1. The linearity of the kinetic plots is consistent with a first-order process with respect to inhibitor concentrations. The reciprocal of the regression lines of increasing slopes corresponds to an increasing inhibition concentration. The common intersection of all slopes at the ordinate and increase in the apparent K_m value indicates that the pesticide is competitive in nature.

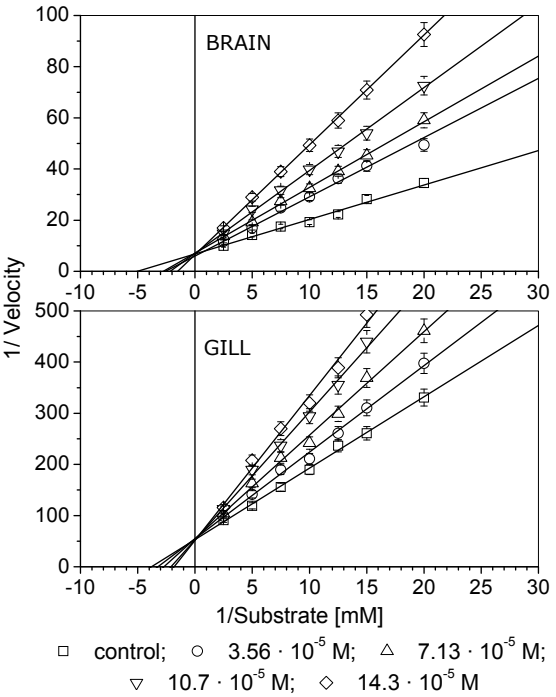

Fig. 1. Lineweaver-Burk (LB) plots of fish brain and gill AChE in the presence of different concentrations of CPP. The graphs were plotted using reciprocals of the relative hydrolysis velocities *versus* reciprocal substrate concentrations of the inhibitor concentrations. Each value is the mean ± SE of three individual observations. Values are significant at $P < 0.05$.

The kinetic constants (V_{max} and K_m) describing the hydrolysis of acetylthiocholine substrate by AChE in fish brain and gill are presented in Tables I and II, respectively. They summarize the apparent K_m value in the presence of increasing concentrations of CPP in both fish brain and gill. CPP reflected a significant increase in K_m with an increase in inhibitor concentration in fish brain when compared to fish gill. It is also evident from the results that CPP inhibited this target enzyme in a concentration- and time-dependent manner. Similarly, it was reported that benthiocarb and monocrotophos inhibited *in vitro* the fish brain AChE activity in a concentration-dependent manner (Babu *et al.*, 1989; Rahman *et al.*, 2004). Earlier findings also suggested that brain AChE activity can be used as a good diagnostic tool for OP and carbamate pollution (Dembele *et al.*,

Table I. Relative V_{max} and percent increase in K_m of fish brain AChE with increase in the CPP concentration.

Concentration [M]	Intercept	Slope	V_{max}	Relative K_m (1/intercept) · slope	
				K_m [mM]	Percent increase
$3.56 \cdot 10^{-5}$	7.033	2.20	0.142	0.314	22.140
$7.13 \cdot 10^{-5}$	6.992	2.58	0.143	0.369	51.361
$10.69 \cdot 10^{-5}$	7.282	3.21	0.137	0.441	71.634
$14.26 \cdot 10^{-5}$	6.702	4.27	0.149	0.637	147.93
Control	6.676	1.71	0.149	0.257	–

Table II. Relative V_{max} and percent increase in K_m of fish gill AChE with increase in the CPP concentration.

Concentration [M]	Intercept	Slope	V_{max}	Relative K_m (1/intercept) · slope	
				K_m [mM]	Percent increase
$3.56 \cdot 10^{-5}$	54.846	16.9	0.019	0.30	15.4
$7.13 \cdot 10^{-5}$	54.724	20.2	0.018	0.37	42.3
$10.69 \cdot 10^{-5}$	52.406	25.1	0.018	0.48	84.6
$14.26 \cdot 10^{-5}$	51.573	28.14	0.019	0.54	107.6
Control	53.166	13.9	0.019	0.26	–

2000). Its inhibition either directly causes or is an indirect indicator of acute central nervous system and peripheral nervous system symptoms (Bakshi *et al.*, 2000).

The inhibition constants were derived from double reciprocal plots of K_m/V_{max} regressed against inhibitor concentrations (Fig. 2). The k_i value was significantly different for both tissues with $7.3 \cdot 10^{-5}$ M and $11.92 \cdot 10^{-5}$ M for brain and gill AChE, respectively, indicating that CPP is a stronger inhibitor of fish brain, in comparison to gill. This may be due to the fact that fish brain contains pure AChE enzyme whereas gill, being a respiratory organ, rich in blood supply, may contain many isoenzymes along with AChE. The bimolecular rate constant (k_i) is generally considered to be the most reliable criterion to evaluate the inhibitory power of OP insecticides to AChEs. Our earlier results showed that CPP is less sensitive to fish brain, *Gambusia affinis*, with a k_i value of $4.57 \cdot 10^{-4}$ M (Kavitha and Venkateswara Rao, 2008), whereas it is highly sensitive to earthworm, *Eisenia foetida*, with a k_i value of $4.2 \cdot 10^{-6}$ M (Venkateswara Rao *et al.*, 2003a). Comparatively, the OP insecticide profenofos has shown k_i values of $2.38 \cdot 10^{-5}$ M and $2.52 \cdot 10^{-6}$ M in fish, *O. mossambicus*, and earthworm, *Eisenia foetida*, respectively, indicating that profenofos is a stronger inhibitor (Venkateswara Rao *et al.*, 2003b; Chakra Reddy and Venkateswara Rao, 2008).

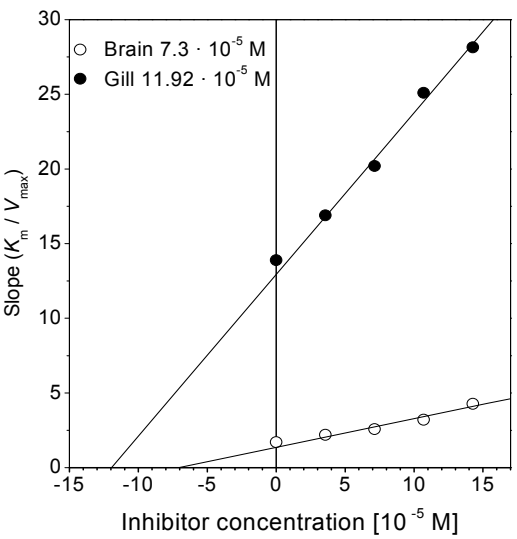

Fig. 2. Inhibitor constant (k_i) values of CPP for euryhaline fish (*Oreochromis mossambicus*) brain and gill AChE.

AChE is considered as a specific biomarker enzyme for OP and carbamate pesticide exposures, being commonly used to diagnose exposure of natural populations to these chemicals. The results of this *in vitro* study show that the kinetic mechanism of CPP-induced AChE inhibition gives the evidences that this action is competitive as evaluated from kinetic constants. Similarly, it was reported that OP pesticides inhibited *in vitro* fish brain by altering the K_m and k_i values (Qadri *et al.*, 1994; Venkateswara Rao *et al.*, 2003b). The results suggest that in biomonitoring programmes, brain AChE activity can be a good diagnostic tool for CPP toxicity, and the present study also points to the importance of kinetic studies.

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