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RESEARCH ARTICLE

Evaluation of Levels of Phosphatases in the Lindane Exposed Fish, *Channa punctatus*

Aradhna Gupta and Bechan Sharma*

Department of Biochemistry, University of Allahabad, India

ABSTRACT

The alterations in the activities of acid and alkaline phosphatase are good indicators of stress due to the exposure of fish to any toxicant. The perturbations in their activities may result in the decreased immune responses and intestinal microflora activity. In the present study, the effect of sublethal concentrations of lindane (0.025, 0.05 and 0.1 mg/l) in different tissues of *C. punctatus* exposed for 96h has been studied. The data showed decrease in the acid phosphatase (AcP) activity in the following order: gills>muscle>heart>brain>kidney>liver, respectively, and for the Alkaline phosphatase (ALP) the order was gills>liver>muscle>heart>brain>kidney, respectively, for 96h treatment. At highest concentration of lindane (0.1 mg/l) tested; the maximum decrease in AcP activity was recorded in gills (47.11%) and minimum in liver (15.02%). The other organs such as muscle, heart, brain and kidney exhibited 38.12, 30.85, 29.97 and 16.85% decrease, respectively. The activity of ALP showed maximum decrease in gills (27.93%) and minimum in kidney (0.98%). At highest concentration of lindane (0.1 mg/l) tested, the brain, heart, liver and muscle registered 1, 6.29, 18.84 and 14.25% decrease in ALP activity, respectively, under this condition.

Introduction

The freshwater organisms act as the sink of both natural and anthropogenic inputs of contaminants into the environment. Fish are essential components of healthy aquatic systems. They are ecologically and economically valuable providing humans with recreation and nutritive food. The release of toxic substances in the environment by either human activities or natural cases have been reported to cause excessive fish mortality [1]. The exposure of aquatic organism to even sublethal concentrations of contaminants may prove to be equally devastating [2]. Pesticides like glyphosate, Deet, Propoxur, Malathion have been exhaustively used in agriculture to combat the pest menace to produce higher yield of crops improving the economy. Among these pesticides, HCH (1,2,3,4,5,6 hexachlorocyclohexane, commonly known as lindane) is the most indiscriminately used insecticide, which has been shown to cause toxicity into the non-target aquatic organisms as well. Due to the non-biodegradable and persistent nature of HCH in the environment, it undergoes biomagnification through food-chain thereby causing serious concern to the human health [3].

The phosphatases (both the acid and alkaline) are known to be active

***Corresponding author(s)**

Bechan Sharma, Department of Biochemistry, University of Allahabad, India

Email: sharmabi@yahoo.com

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at acidic and alkaline pH ranges, respectively, and are usually termed as phosphomonoesterases. The changes in the activities of these enzymes due to pesticide exposure in the fish tissues have been reported as indicators of pesticide toxicity [4-6].

Alkaline phosphatase (ALP) is an enzyme that is found in various tissues throughout the body, such as the liver, bones, and intestines. It plays a crucial role in the metabolism of various compounds in the body, including proteins, carbohydrate metabolism, transphosphorylation reactions, secretary activity and nucleotides metabolism [7]. It affects the intestinal microbiota, causes systemic inflammation inhibiting tissue infiltration of neutrophils at the site of inflammation and induces nutrient absorption like calcium, phosphorus, fatty acids. Tissue non-specific alkaline phosphatase (TNAP) is a form of enzyme found in various tissues throughout the body, including bone, liver, kidney, and intestine. It is also involved in various other physiological processes, such as cell signaling and lipid metabolism, and abnormalities. Its expression or activity has been linked to a range of other medical conditions, including liver disease, kidney disease, and cancer. Studies have proved that it is also present in fish mucus and protects it from water-borne pathogens, detoxify proinflammatory barriers by dephosphorylation like lipopolysaccharides, and hence not recognised by their specific receptors to show any proinflammatory response involving NF-κB activation pathway, gene activation and release of pro-inflammatory cytokines (IL-8) [8]. Its increased activity has been associated with the colonic tissue inflammation [9].

Acid phosphatase (AcP) is a lysosomal enzyme surrounded by lipoproteins that hydrolyses the phosphorous esters in acidic medium. AcP is involved in autolytic process of the cell after its death [10].

The impact of organochlorine group of pesticides on the activities of phosphatases (AcP and TNAP) in different tissues of the aquatic organisms including the fresh water fish, *Channa punctatus,* has not been properly studied. The present study, was therefore carried out by exposing *C. punctatus* with different sublethal concentrations of lindane for 96hr and evaluating the impact of the pesticide on the levels of phosphatases in the fish tissues.

Materials and Methods

Lindane was procured from Rallis India Ltd. Bangalore, India and was dissolved in acetone (AR grade) for use. All other reagents used in this study were analytical grade.

The teleost fish, *C. punctatus* (length 6.0 cm to 8.0 cm and weight 25-30 g) were procured from the local fish market. The fish were thoroughly washed with running tap water and acclimatised in aquatic environment for 7 days. The fish were divided in four groups each containing 10 fish to be used as (i) control, treated with (ii) 0.025 mg/l, (iii) 0.05 mg/l and (iv) 0.1 mg/l lindane. Another group of fish treated with the corresponding concentration of acetone has been used in our earlier studies and it did not exert any adverse effect and the results were comparable to that of control (i).

C. punctatus, treated with different sublethal concentrations of lindane (0.025, 0.05 and 0.1 mg/l) were sacrificed after the stipulated treatment period i.e. 96h and its different body organs such as brain, gills, heart, kidney, liver and muscles were dissected out for estimation of protein and assay of enzyme activites.

Tissue homogenization

The tissues were homogenised to prepare their homogenates (10%, w/v) in normal saline under cold conditions. The homogenates of different fish tissues were centrifuged at 10,000 xg at 6°C and the supernatants were collected for estimations of different biochemical parameters.

Protein determination

The quantitative estimation of total protein in various tissue extracts were done according to Lowry OH, et al. [11]. The Bovine Serum Albumin (BSA) was used as standard. A blank was prepared which contained all reagents but no protein. The intensity of blue colour was measured calorimetrically at 620 nm.

Assays of the activities of acid and alkaline phosphatases

The activities of Acid and alkaline phosphatases (AcP, EC 3.1.3.2; ALP, EC 3.1.3.2) were assayed by the method of Salomon LL, et al. [12]. For assay of AcP activity, the reaction mixture (3.0 ml) contained sodium acetate buffer (100 mM, pH 4.5), p-nitrophenyl phosphate (2.5 mM) and cell-free extract (100-200 µg protein). The mixture was incubated for 20 min at 37°C with intermittent gentle shaking. The reaction was terminated by addition of

NaOH (2.0 ml, 1 N). The intensity of yellow colour was measured calorimetrically at λ_{max} 405 nm.

For assay of ALP, the reaction mixture contained p-nitrophenyl phosphate (2.0 mM) prepared in bicarbonate buffer (50 mM, pH 9.5) and cell free extract (100-200 µg protein). The mixture was incubated for 30 min at 37°C with intermittent gentle shaking. The reaction was stopped by addition of NaOH (0.4 ml, 0.1 N) which developed yellow colour. The intensity of yellow colour was measured calorimetrically at λ_{max} 410 nm. The Optical Density (OD) observed with the control was corrected from the experimental observations. The p-nitrophenyl phosphate was used as standard.

The activity of AcP was calculated as following: ΔOD x 621.1/0.24 x incubation time (min) x protein (mg) and the unit was expressed as nanomoles p-nitrophenol formed min^{-1} mg^{-1} protein. Where 621.1 nM p-nitrophenol corresponds to OD equal to 0.24 at λ_{max} 405 nm.

Similarly, the activity of ALP was calculated as following: ΔOD x 621.1/0.36 x incubation time (min) x protein (mg) and the unit was expressed as nanomoles p-nitrophenol formed min^{-1} mg^{-1} protein. Where 621.1 nM p-nitrophenol corresponds to OD equal to 0.36 at λ_{max} 410nm. Spectrophotometric measurements were made using UV-VIS spectrophotometer with silica glass/quartz cuvettes (1 cm light- path, capacity 3 ml) against enzyme blank containing all the reactants at room temperature.

Results and Discussion

The effects of lindane on the activities of AcP and ALP in different organs of *C. punctatus* were monitored according to the procedure as mentioned in Materials and Methods. The results reflecting the impact of the pesticide on the enzymes' activities are shown in tables 1,2.

The data shows highest activity of AcP in kidney (50.55 ± 2.89 units/mg protein) and lowest in gills (18.36 ± 3.47 units/mg protein) of the control fish. The values in other tissues such as in liver, heart, muscle and brain were recorded to be 44.53 ± 2.33, 38.116 ± 1.73, 27.41 ± 4.15, 20.34 ± 2.32 units/mg protein, respectively (Table 1). The effect of lindane was more prominent at higher concentration (0.1 mg/l) than at lower concentrations (0.025, 0.05 mg/l) for 96h. At lowest concentration (0.025 mg/l), the activity of AcP was not affected in liver, whereas brain, heart and muscles displayed around 15% inhibition in the enzyme activity. Under this condition, kidney and gills showed about 5 and 20% reduction in the enzyme activity, respectively. This data indicated that at lowest concentration of the pesticide, gills were maximally affected whereas liver showed complete resistance towards pesticide toxicity. At the highest concentration of lindane (0.1 mg/l) the maximum decrease in AcP activity was recorded in gills (47.11%) and minimum in liver (15.02%). The other organs such as muscle, heart, brain and kidney exhibited 38.12, 30.85, 29.97 and 16.85% decrease in enzyme activity, respectively. Under this condition, the order of diminution in the activity of acid phosphatase in

Table 1: Effect of Lindane on the specific activity of acid phosphatase in different tissues of *C. punctatus* exposed for 96h.

Activity of Acid Phosphatase (Units/mg protein)				
	Lindane (mg/l)			
Organ	0	0.025	0.05	0.1
Brain	20.34 ± 2.32	17.25 ± 1.15* (-15.23)	17.19 ± 1.73* (-12.33)	14.23 ± 3.47* (-29.97)
Gills	18.36 ± 3.47	14.72 ± 4.05 (-19.84)	11.38 ± 1.16* (-37.96)	9.70 ± 2.54* (-47.11)
Heart	38.116 ± 1.73	32.34 ± 1.74 (-15.11)	30.86 ± 2.87 (-18.96)	26.40 ± 3.45* (-30.85)
Kidney	50.55 ± 2.89	47.95 ± 2.31* (-4.96)	44.27 ± 2.29* (-12.42)	42.03 ± 1.15* (-16.85)
Liver	44.53 ± 2.33	42.44 ± 2.31 (-4.7)	39.08 ± 2.32 (-12.26)	37.86 ± 4.04 (-15.02)
Muscle	27.41 ± 4.15	23.42 ± 1.85 (-14.57)	18.84 ± 1.18 (-31.24)	16.96 ± 1.18* (-38.12)

Values are presented as nM p-nitrophenol formed min^{-1} mg^{-1} protein. Each value represents the mean± SEM of three different observations. Values in parenthesis are percent change over control. The (-) sign represents decrease over control. Significance of data is shown in superscripts. Significantly different from control at $^{**}p < 0.01$, $^{*}p < 0.05$ (Student's t test). h: hour; SEM: Standard Error of Mean.

different tissues of the fish was recorded as following: gills>muscle>heart> brain>kidney>liver. The data indicated that at highest concentration (0.1 mg/l), gills displayed maximum sensitivity to lindane whereas the liver was affected to minimum possible extent (Table 1).

The results of the effect of sublethal concentrations of lindane (0.025, 0.05 and 0.1 mg/l) on the activity of ALP in different tissues of *C. punctatus* is shown in table 2. The data demonstrated highest activity of ALP to be present in kidney (82.03 ± 1.49 units/mg protein) and lowest in brain (30.4 ± 2.88 units/mg protein) of the control fish. The activities of ALP in other fish tissues such as liver, heart, muscle and gills were recorded as 70.84 ± 2.92, 53.60 ± 2.26, 4.23 ± 4.44 and 36.82 ± 3.45 units/mg protein, respectively. At the highest concentration of lindane treatment, the order of decrease in the activity of ALP in different fish tissues was as follows: gills>liver>muscle>heart >brain>kidney (Table 2).

The comparison of the data presented in tables 1,2 indicated that lindane at subacute concentrations caused marked decrease in the activities of enzymes in all the organs of the fish tested. In both of the cases, the inhibitory effect of lindane was concentration dependent and organ specific. However, the effect was more marked on the AcP as compared to ALP. Lindane caused sharp decrease in the activity of AcP and ALP in the gills of the fish at highest concentration of lindane (0.1 mg/l). The activity of AcP was least affected in liver whereas the activity of ALP was least affected in kidney of the fish exposed to the pesticide for 96h.

The activities of AcP and ALP are good indicators of stress of a toxicant on fish [13]. Any damage or dysfunction in experimental organs is manifestation of alteration in phosphatase activity [14]. The reduction in the activities of these enzymes could be due to increased utilization of protein to meet out the increased energy cost of homeostasis, tissue repair and detoxification during stress [15,16].

The decrease in AcP activity correlates its role in cellular activity and cellular damage leading to autolytic breakdown or cellular necrosis resulting in insufficient production of enzymes or leakage of enzyme into extracellular compartment due to pesticide treatment [17]. Necrotic changes in different organs might be responsible for increase or decrease in the activity of phosphatases [18-20]. The altered enzyme activities can be due to factors that influence the rate at which enzyme enters the circulation from the cells or rate of enzyme production by individual cell type or proliferation of a particular type of enzyme producing cell. It is generally accepted that an increase or decrease in the activity of enzymes in the extracellular fluid or plasma is a sensitive indicator of minor cellular damage [21].

It was observed in *Cyprinus carpio* that a herbicide, 2,4 diamin at sublethal concentrations (50 and 80 ppm) did not cause any significant alteration in the activities of both phosphatases after different exposure period (1-30 days) and they have proposed that this herbicide does not cause destruction of cell membranes and lysosomes, which could liberate their hydrolases into the cell system of tissues to

Table 2: Effect of Lindane on the specific activity of alkaline phosphatase in different tissues of *C. punctatus* exposed for 96h

Activity of Acid Phosphatase (Units/mg protein)				
	Lindane (mg/l)			
Organ	0	0.025	0.05	0.1
Brain	30.4 ± 2.88	35.5 ± 3.78* (17)	33.7 ± 2.36 (11)	30.09 ± 2.86 (-1)
Gills	36.82 ± 3.45	34.80 ± 2.42 (-5.48)	30.62 ± 2.86 (-16.78)	26.56 ± 2.03 (-27.93)
Heart	53.60 ± 2.26	56.145 ± 4.05* (4.75)	57.50 ± 1.23* (7.29)	50.22 ± 2.86 (-6.29)
Kidney	82.03 ± 1.49	80.02 ± 3.28 (-2.45)	83.28 ± 2.60 (1.52)	81.23 ± 3.20 (-0.98)
Liver	70.84 ± 2.92	67.96 ± 2.23 (-3.99)	58.22 ± 1.84* (-17.78)	57.50 ± 4.14* (-18.84)
Muscle	47.23 ± 4.44	44.19 ± 4.36 (-6.42)	42.30 ± 2.03* (-9.42)	40.48 ± 2.88* (-14.25)

Values are represented as nM p-nitrophenol formed min^{-1} mg^{-1}protein. Each value represents the mean± SEM of three different observations. Values in parenthesis are percent change over control. The (-) sign represents decrease over control. Significance of data is shown in superscripts. Significantly different from control at $^{**}p < 0.01$, $^{*}p < 0.05$ (Student's t test). h: hour; SEM: Standard Error of Mean.

facilitate autolysis [22]. The decrease in activity of AcP in the tissues of *C. punctatus* exposed to sublethal concentrations of lindane in present investigation is supported by the results obtained with *C. gachua* exposed to thiodan and rogan for 30 days [23]. A reduction in the gill AcP activity was reported in rosy barb, *Puntius conchonius* (Hamilton) exposed to copper and cadmium toxicity [24], in the ovary and liver of *C. punctatus* exposed to zinc (10-25 mg/l) for 8, 10 and 15 days [25], in *C. punctatus* exposed to monocotrophs [26], and in *Clarius gariepinus* exposed to aqueous leaf extract of *Lepidhagathis alopecuroides* [27].

Apart from pesticides, the sharp decline in the activities of AcP and ALP has been reported by many workers for example in *H. fossilis* treated with cadmium; *Sarotherodon mossambicus* with mercury [28]; in *Labeo rohita* with copper, and zinc [29] and in rainbow trout (*O. Mykiss*) with chromium [30]. However, *Perna viridis* has been shown to get affected by the exposure to copper, lead and zinc causing elevation in the ALP activity [31].

The decrease in activity of ALP in the present study could be due to uncoupling of oxidative phosphorylation. Its activity has been shown to be reduced by 56% in Crucian carp (*Carassius carassius*) [32] when water temperature changed from18˚C to 2˚C. The use of alkaline water with pH 8.0-7.5 has resulted into decreased activity by 22% at 22˚C in Flat fish larvae (*S. senegalensis*) [33], and in the liver of *Sarotherodon mossambicus* [34]. The acidic water may inactivate ALP to get energy *via* anaerobic breakdown of glycogen. In saline water its activity was found to be increased in juveniles of *Alosa sapidissima* [35]. Its activity in general was found to be higher in summer and lower in winter in Crucian carp (*C. carassius*) [36]. However, the impact of physico chemical properties of water on the activity needs more study to reach to any conclusion. It may also depend upon food intake, oxygen concentration of water; season, and the place from where the fish has been taken etc. Several studies have reported increased enzyme activity in fish blood due to the leakage from liver [37,38]. The reduction in the ALP activity in the brain may be attributable to the alteration in the nucleic acid content because of pollutants. A statistically significant decline in Acp and ALP activities during 30 days exposure of fish to pesticide has been reported [39]. In contrast to our findings some workers have reported the increase in the activities of AcP and ALP, which has been attributed to onset of hepatic disorder and inflammatory reactions etc. [40-44].

Conclusion

The data suggests that lindane even at the very small concentration was able to pose threats to the life of *C. punctatus.* The sub-lethal concentrations of lindane caused perturbations in the levels of phosphatases in all the organs of the fish but maximum was in gills of the fish exposed for 96h. The perturbations in the levels of phosphatases may be used as early signals for assessment of toxic effects of the pesticides in aquatic system. The results from this study may be useful towards adequate formulation of pesticides, and policy makers for the sustainable management of ecophysiological and the environmental health of aquatic animals. These results also may offer a sound understanding of the toxicological endpoint of the aquatic life. Further research is needed to fully understand the effects of lindane on fish and other organisms and their potential implications for the environment and human health.

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Conflicts of Interest

There is no conflict of interest to be disclosed.

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