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Effect of heavy metals on Digestive enzymes protease and invertase of Bivalve L. marginalis…..

*Sandhya M. Sonawane.
Department of Zoology, Nutan Maratha College, Jalgaon, Dist. Jalgaon. (M.S.) Mobile No. 9422977824
Corresponding Author: Sandhya M. Sonawane.

Abstract: *Enzymes are referred as biological, catalysts which obey certain general rules. . The literature shows investigative lacunae with regard to effects of heavy metals on digestive processes of aquatic animals especially where fresh water molluscs are concerned. Hence an attempt has been made to study the effect of heavy metals on fresh water bivalve, Lamellidens Marginalis with respect to change in the level of digestive enzymes. In the present investigation invertase and protease activity was significantly inhibited in Lamellidens Marginalis after acute and chronic exposure of heavy metals, $CuSO_4$, $HgCl_2$ and $CdCl_2$. However, among the tested heavy metals $HgCl_2$ was found to be the potent inhibitor of invertase activity.*

Keywords*: Heavy metals, L. marginalis, protease, invertase, acute, chronic.*

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I. INTRODUCTION

Enzymes are referred as biological, catalysts which obey certain general rules. The enzyme catalyzed reactions take place at physiologically low temperatures (37^0C) and require extremely small amounts of enzymes. Chemically, enzymes are complex protein molecules synthesized in the cells where they act as biocatalysts in carrying out various physic-chemical reactions. These proteins have their own specificity and kinetics. The enzymes help in attaining a reaction in a state of equilibrium. An enzyme recognizes its specific substrate and reacts with it to form products and gets regenerated at the end of the reaction.

The usual measure applied for the assessment of any environmental effect of a pollutant on animal is mortality. However, other effects which are gradual and are indicative of physiological change can be as detrimental as mortality to the animal survival. Bioabsorption and bioaccumulation of heavy metals by rock oyster. (H.,A.J.Khoi, A.Mirvaghefi, A.Danekar and M.Shapoori. 2010)

Although instantaneous effects of heavy metal poisoning may be physical such as retraction of animal in the shell, loss of locomotory activity, changes is normal behavior, reproductive disorders, suffocation, coating of respiratory surface with mucus, long term effect would be exclusively due to physiological alterations. The most fundamental effects would be the study of changes in enzyme levels, since these organic cellular catalysts control the formation of biochemical intermediates which are indispensable to all normal physiological processes.Toxicants in aqueos ecosystems: a guide for the analytical and environmental chemist, New York(Crompton, T.R.2006). Molluscs have exhibited ability to adapt themselves to life in so many different types of habitats. It is not surprising that they have learnt to feed in different ways. They show a variety of digestive patterns. The correlation between digestive enzymes and diet has been established but specific characterization of different enzymes of different animals presents many interesting and puzzling questions (Prosser 1973). Many workers (Mukherji and Kanungo, 1954) have investigated the digestive enzymes of pelecypods. They were reported from digestive diverticula and digestive gland of several lamellibranchs.

Heavy metals are pervasive components of the aquatic environment, including fresh waters. The gross effects of these components have been substantially documented in both marine and fresh water. Yet the extent of physiological damage resulting from high and low level heavy metal contamination of fresh water and the harm that is done to aquatic animals, has not been adequately described, though there are few studies which indicate the effect of heavy metals on aquatic organisms (Hewitt and Nicholas, 1963). A change in enzymatic pathway is another approach which though yet untried could potentially be used in toxicant analysis. Exposure to metals might in some way alter the enzyme and change its response in co-factors, temperature, pH and also its micheli's constant (Jackim, 1974).

The mollusc have a surprising enzyme equipment. Indeed there seems to be no other group in the animal kingdom with such an array of digestive enzymes. Particularly the carbohydrases. Studies on the digestive enzymes of lamellibranches were first initiated by Young (1926) in oyster. Mansour and Zaki (1946) and Zaki (1951) reported the presence of proteases, peptidases and lipases from the stomach juice as well as digestice diverticula of Unio Prasedens. Mukherji and Kanungo (1954) reported the presence of invertase in the

digestice gland of common Indian fresh water Pelecypod Lamellidens. But among the fresh water mussels little work has been done on the physiology of digestion and digestive enzymes. Hence to study the changes in enzymatic pathways it is a potent approach to assess the toxicity of the heavy metals. Therefore by measuring the activity of some key enzymes it is possible to determine the physiological effects of the heavy metals on the biological systems. Kreb's citric acid cycle is the final and common pathway for the oxidation of carbohydrates, proteins, lipids, since glucose, amino acids and fatty acids are metabolized to acetyl Co-A and then acetyl Co-A is oxidized to Co_2 and water through a series of metabolic steps. The oxidation of acetyl Co-A reduces equivalents in the form of electrons. These electrons are released due to activity of a group of specific enzymes known as dehydrogenases. This dehydrogenase catalyses the formation of high energy phosphate bond through a process of oxidative phosphorylation (Campbell, 1973). The energy, thus generated is most important for the organism. Any disruption or alteration in the activity of these enzymes of the citric acid cycle, may therefore, disturb the entire physiological equilibrium resulting in complications of various nature.

Poison of any nature, whether pesticides, salts of heavy metals can cause ultrastructural change in the mitochondria, endoplasmic reticulum etc. and inhabit the enzymes of TCA cycle. Kabeer Ahmed (1979) in the snail, Pila Globosa observed changes in oxidative metabolism after exposure to organophosphate pesticide. Alam (1984) in the snail. V. Bengalensis observed alteration in oxidative metabolism after exposure to salts of heavy metals like Cu, Hg and Zn. Shellfish aquaculture and the environment, (Shumway,S.E.2012) . For going survey of literature showed that only a few workers have studied digestive enzymes, respiratory and metabolic enzymes of lamellibranch mollusks. The literature shows investigative lacunae with regard to effects of heavy metals on digestive processes of aquatic animals especially where fresh water molluscs are concerned. Hence an attempt has been made to study the effect of heavy metals on fresh water bivalve, Lamellidens Marginalis with respect to change in the level of digestive and metabolic enzymes.

II. MATERIAL AND METHODS

The bivalves Lamellidens Marginalis were collected from the Godavari river at Paithan. After bringing the bivalves to the laboratory, they were cleaned thoroughly and placed in plastic troughs. They were acclimatized to the laboratory conditions for 5 to 6 days prior to subjecting them to experiments. The water in the troughs was changed every day. Only active and healthy animals were chosen for experiments. During chronic treatment the animals were fed on crushed fresh water algae and Hydrilla. The bivalve were exposed to median lethal concentration and sublethal concentration of popputant as acute and chronic treatment respectively.

The acclimatized bivalves were divided into four groups, of ten each. The first groups of bivalves was kept as control. The remaining groups were exposed to 1.6 ppm $Cuso_4$, 0.6 ppm $HgCl_2$ and 3.9 ppm cadmium chloride for 72 hours, for acute treatment. The concentrations used for chronic exposure were 0.82 ppm copper sulphate, 0.32 ppm mercuric chloride and 1.95 ppm cadmium chloride. The chronic treatment was given upto 20 days. The control and treated bivalves were fed on freshwater crushed algae and Hydrilla during exposure period. The digestive glands from five to ten mussels were separated and washed in distilled water. These digestive glands were then dried between the folds of muslin cloth, dehydrated and defatted by treatment with ice cold acetone (Summer and Summer, 1947). The material was ground in a clean ice chilled glass morter and repeatedly washed in acetone and filtered till and filtrate was colourless. The powder thus obtained was dried under fan and stored in a clean bottle in freezer, at 3 to 5^0C. In all the experiments, 1% homogenate of digestive gland prepared in glass distilled water was used. Half of this extract was boiled for half an hour to destroy the enzyme activity and this boiled extract was used as control for all the experiments.

Qualitative assay of digestive enzymes in digestive gland :

Action of digestive gland enzymes on carbohydrates was determined by carrying out experiments with the substrates such as sucrose, maltose lactose, carboxymethyl cellulose, 10 ml of 1% digestive gland extract, experimental and control were taken in separate flask. Then 10 ml of 1% different substrates and 5 ml of buffer solution were added separately. The reaction mixture was incubated for 24 hours at 30^0C. After incubation to each enzyme mixture, fehling's A and Fehling's B solution were added in equal volumes. The mixture was subjected to heating for 10 minutes and allowed to stand for few minutes. Appearance of red precipitate indicates the presence of invertase, maltase, lactase and cellulase.

Test for Invertase:

To 5 ml of extract, 5 ml of 1% sucrose were added. The mixture was incubated for 20 hours at 30^0C. After incubation a drop of fehlings A and then a drop of fehlings B were added to the mixture A red precipitate appears indicating the presence of invertase.

Test for Protease:

To 5 ml of extract, 4 ml of 1% peptone were added. The mixture was incubated for 20 hours at 30^0C. After incubation a drop of phenolphthalein was added which developed pink colour. Disappearance of pink colour after some time indicated the presence of protease.

III. ENZYME DETERMINATION METHODS

Estimation of invertase:

The invertase activity was determined as described by Noelting and Bernfoold (1948). The reaction mixture consists of 0.5 ml substrate (2%), 1.5 ml phosphate buffer (pH 7.5) and 0.5 ml tissue homogenate (16% W/V). After 1 hr of incubation at 37^0C, the enzyme activity was terminated by adding 2 ml of 3.5 dinitrosalicylic acid reagent, the heated on boiling water bath for 5 minutes, allowed to cool and the optical density was recorded at 5.30 um. For invertase activity sucrose solution was used as a substrate. A known amount of maltose and glucose with the same procedure gave the calibration curve for estimating amylase and invertase activity respectively.

Estimation of protease:

Protease activity was determined by following sorenson's formaldehyde titration method as modified by prosser and Vanweel (1958). The reaction mixture consists of 0.3 ml Gelatin (3%) 1 ml phosphate buffer (pH 7.5) and 1.0 ml tissue homogenate (10% W/V). After the incubation of 60 minutes at 37^0C, the enzyme activity was terminated by keeping it in boiling water bath for 5 minutes. The equal amount of neutral formaldehyde was added and titrated against 0.1 N KOH solution. 0.5% alcoholic phenolphthalein was used as an indicator. The initially boiled tissue homogenate with the same procedure served as control. The difference between the experimental and control gave the amount of protease activity. The amount of amino acid liberated in terms of 1 ml of 0.1N KOH was taken as an index of enzyme activity.

IV. OBSERVATIONS AND RESULTS

The activities of invertase and protease enzymes were studied in the normally fed and pollutant (heavy metals) treated freshwater bivalve, Lamellidens marginalis. The experimental findings obtained are summarized in Tables 1 to 4 Digestive enzymes (Acute and chronic exposure to heavy metals $CuSO_4$, $HgCl_2$ and $CdCl_2$

Invertase:

The invertase activity in control animals was 0.0982 mg of glucose/mg protein/hr. In case of $CuSO_4$ the invertase activity increased at 48 hours exposure while $HgCl_2$ showed a continuous decrease in activity at 24, 48 and 72 hours of exposure. The values were 0.0428, 0.0400 and 0.0370 mg at 24, 48 and 72 hours respectively. In case of $CdCl_2$ the invertase activity decreased which was 0.0921, 0.0825 and 0.0720 mg of glucose/mg protein/hr at 24, 48 and 72 hours respectively. The increase and decrease in activity in $CuSO_4$ and $CdCl_2$ stress were not significant at 24, 48 and 72 hours, while decrease in activity in $HgCl_2$ stress was significant at $P<0.001$ and $P<0.05$ levels. The maximum percentage decrease was upto 72.10% after 72 hrs exposure to mercuric chloride and minimum percentage increase was upto 1.095% (24 hrs. $CdCl_2$ stress). Changes in invertase activity are summarized in table 1. The control animals showed 0.2002 mg glucose/mg protein/hr. A constant depletion in invertase activity was observed after the stress of heavy metals. HgCl2 stress decreased the activity more than $CuSO_4$ and $CdCl_2$ stress decreased the activity more than $CuSO_4$ and $CdCl_2$. The values of invertase activity were 0.0602, 0.0101 and 0.0762 mg of glucose/mg protein/hr at the end of 20 days. All values are statistically significant at $P<0.01$ and $P<0.001$ levels. The maximum percentage decrease was upto 82.20% at the end of 20 days at $HgCl_2$ stress.

Portease:

The protease activity in the control bivalves,: L. Marginalis was found to be 8.003 mg of amino acids / mg protein / hr at room temperature, $HgCl_2$ was found to be more potent in inhibiting protease activity. $CuSO_4$, $HgCl_2$ and $CdCl_2$ showed protease activity of 3.200, 2.002 and 4.030 mg of amino acids/mg protein/hr respectively after 72 hours. The decrease in protease activity at 24, 48 and 72 hours in all heavy metals is statistically significant at $P<0.001$ and $P<0.05$ levels. The maximum percentage decrease was upto 75.35% (72 hrs of $HgCl_2$ stress). Changes in protease activity after chronic exposure are summarized in Table 4. The control animals showed protease activity of 8.930 mg of amino acids/mg protein/hr. In the present investigation $HgCl_2$ stress showed more decrease in protease activity than $CuSO_4$ and $CdCl_2$ after chronic exposure. The values were 4.432, 2.813 and 5.303 mg of amino acids/mg protein/hr for $CuSO_4$, $HgCl_2$ and $CdCl_2$ respectively at the end of 20 days. Among the tested heavy metals $CdCl_2$ showed less decrease in protease activity at 5, 10, 15 and 20 days than $CuSO_4$ and $HgCl_2$. All the values were statistically significant at $P<0.001$ and $P<0.01$ levels. The maximum percentage decrease was upto 72.13% after 20 days of $HgCl_2$ exposure.

Table – 1 Changes in the invertase activity in the bivalve L. marginalis after acute exposure of heavy metals Copper sulphate, Mercuric chloride and Cadmium chloride

Sr. No.	Control	Copper Sulphate			Mercuric Chloride			Cadmium Chloride		
1.	0.0982± 0.0008	0.0901± 0.039	0.0931± 0.031	0.0820± 0.030	0.0428± 0.038	0.0400± 0.035	0.0370± 0.032	0.0921± 0.039	0.0825± 0.021	0.0720± 0.042
		N.S.	N.S.	N.S.	P< .05	P< .001	P<0.05	N.S.	N.S.	N.S.
		-14.20	+0.9020	-41.70	-60.40	-70.10	-72.65	+1.095	-12.30	-20.80

Each activity is expressed as mg. of glucose/gm of protein/hr.
Each value is the mean of five observation ± S.D.
Values are significant at P< .05, P< .001 or NS = Not significant
* These values indicates % stimulation (+ve) or % inhibition (-ve)

Table – 2 Changes in the protease activity of the bivalve L. marginalis after acute exposure of the heavy metals Copper sulphate, Mercuric chloride and Cadmium chloride

Sr. No.	Control	Copper Sulphate			Mercuric Chloride			Cadmium Chloride		
1.	8.003± 0.001	4.032± 0.012	3.502± 0.010	3.200± 0.007	3.302± 0.005	2.832± 0.06	2.002± 0.03	5.303± 0.030	5.002± 0.020	4.030± 0.08
		P<0.05	P<0.01	P<0.05	P<0.05	P<0.01	P<0.05	P<0.05	P<0.01	P<0.05
		-60.30	-70.30	-70.61	-70.11	-71.02	-75.35	-33.16	-40.20	-47.27

Protease activity is expressed as Mg. of amino acids/mg protein/hr
Each value is the mean of five observation ± S.D.
Values are significant at P< 0.01, P< .05
* These values indicates percentage inhibition (-ve)

Table – 3 Changes in the Invertase activity of the bivalve, L. marginalis after chronic exposure of heavy metals Copper sulphate, Mercuric chloride and Cadmium chlorides

Enzyme	Control	Copper Sulphate				Mercuric Chloride				Cadmium Chloride			
		Days				Days				Days			
		5	10	15	20	5	10	15	20	5	10	15	20
Invertase	0.2002± 0.0023	0.0998± 0.0020	0.0875± 0.0025	0.0632± 0.0020	0.0602± 0.0003	0.0321± 0.0003	0.0219± 0.0002	0.0109± 0.0001	0.0101± 0.0003	0.0896± 0.0001	0.0862± 0.0002	0.0792± 0.0025	0.0762± 0.0024
		P<0.001	P<0.001	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	P<0.001	P<0.001	P<0.001	P<0.05
		-22.02	-37.30	-40.70	-48.13	-69.70	-75.80	-80.20	-82.20	-3.50	-6.70	-17.20	-25.30

Enzyme activity expressed as mg. of glucose/mg protein/hr
Each value is the mean of five observations.
Values are significant at P<0.001, P<0.05 level.
-ve sign indicates % inhibition

Table – 4 Changes in the protease activity of the bivalve, L. marginalis after chronic exposure of heavy metals Copper sulphate, Mercuric chloride and Cadmium chlorides.

Enzyme	Control	Copper Sulphate				Mercuric Chloride				Cadmium Chloride			
		Days				Days				Days			
		5	10	15	20	5	10	15	20	5	10	15	20
Protease	8.930 ± 0.0042	4.942 ± 0.0034	4.667 ± 0.0042	4.560 ± 0.0032	4.432 ± 0.0030	3.617 ± 0.0050	3.413 ± 0.0037	3.333 ± 0.0027	2.813 ± 0.0043	6.312 ± 0.0032	6.289 ± 0.0042	5.630 ± 0.0033	5.303 ± 0.0014
		P<0.001	P<0.05	P<0.001	P<0.001	P<0.05	P<0.005	P<0.05	P<0.05	P<0.05	P<0.05	P<0.001	P<0.001
		-50.20	-54.60	-55.27	-59.72	-67.29	-68.80	-70.80	-72.13	-47.39	-49.69	-50.07	-51.20

Each activity is expressed as mg. of amino acid/mg. protein/hr
Each value is a mean of five observations.
Values are significant at $P<0.05$, $P<0.001$ level.
a. -ve sign indicates % inhibition .

V. DISCUSSION

Digestion is a sequential process which renders food absorbable through the gut wall by breaking the food into simple molecular components through enzymatic action, such enzymes are secreted by specialized cells at digestive tract. Digestion although dependent on enzymatic action is determined by the functional organization and the structure of digestive system. Out of the many devices which can be applied to investigate the physiological alterations made by the pesticidal and heavy metal treatment, the most fundamental one would be the study of changes in the enzyme activities, since these organic cellular catalysts control the formation of biochemical intermediates which are indispensible to all the normal physiological processes. Recently it was considered that digestion in molluscs was wholly intracellular, by way of phagocytosis or pinocytosis, proceeding specially in the digestive gland. The presence of digestive enzymes in the extract of digestive diverticula of molluscs was first shown by Fredrucq (1878) who found protease in Mya and Mytilus. A number of digestive enzymes were found to occur in the digestive gland particularly, amylase, maltase, lactase, esterase, lipase, protease (Mansourbek, 1954; Vonk, 1960; Vanwell, 1961; Arvy, 1969 and Vanwell, 1970).

In molluscs (Owen, 1968) digestion takes place extracellularly in the gut lumen by the action of digestive enzymes secreted by salivary glands and the enzymes juice that may flow from hepatic channels into the gut lumen. Gut epithelium is, however, poorly supplied with secretory cells. They are chiefly mucous secretory cells. The spheres that are to be phagocytized are first coated with the mucous layer so that they can easily be taken by the digestive cells of digestive gland. The relation importance of the initial extra cellular digestion, however, varies considerably. The carbohydrate digesting enzymes are less, fully investigated in the invertebrates than the vertebrates although amylase and disaccaridases have been shown to be extremely widely distributed. Many pelecypods and gastropods feed on phytoplankton or larger aquatic plants and in those possessing crystalline style, the only extra cellular digestion is of starch, as in mussles and oysters.

There is no doubt that the effect of certain metals is profound on the enzyme activity in aquatic organisms though the mode of action of these heavy metals has not been clearly outlined with regard to their mechanism of action in certain key enzymes which are responsible for the general energetic of animals. There is a considerable amount of literature devoted to the study of organic pesticides concerning the enzyme system of various animals (Yap et al., 1975; Koundinya and Ramamurthi, 1978; Natrajan, 1981; Shastry and Malik, 1981 and Dalela et al., 1982). However, information regarding the effect of heavy metals on the enzyme action is restricted (Hewitt and Nicholas 1963 and Jackim, 1974). In the present investigation invertase activity was significantly inhibited in Lamellidens Marginalis after acute and chronic exposure of heavy metal salts, $CuSO_4$, $HgCl_2$ and $CdCl_2$

However, among the tested heavy metals $HgCl_2$ was found to be the potent inhibitor of invertase activity. The results of the present investigation are in harmony with the results of the earlier workers (Lomte and Patil 1985, 1988; Alam 1981; Nulina Sundari, et al., 1986) Similar observations were also reported in the army worm Mythimna (Pseudaletia) separate by Patil (1986) after administration of different pesticides. He further reported that the changes in the amylase activity were type, dose and time dependent, starvation stress decreased the protease activity which was reported by Mandal and Roy (1981) in the insect Schizodactylus Monstrosus. Chhatoraj and Mull (1969) reported the presence of invertase in the mature larvae of Marasmia trapezalis Verma and Balyan (1972) also noted the presence of invertase and other digestive enzymes in the bollworm, Platyedra gossypiella. Sinha (1976) studied the invertase activity in the midgut of Musca domestica and Sarcophaga ruticornis. Pesticidal impact caused depletion in the invertase activity in the army worm M. (P.) Separata (Patil, 1986) Nulind Sundari et al. (1986) reported depletion in the amylase, invertase and protease activity in Spodoptera Litura when exposed to Vinca Rosea extract. A significant decrease in invertase activity was noted by Lomte and Patil (1985, 1988) in the army worm M. (P.) separate. Similar observations have been reported by Alam (1984) in Viviparous Bengalensis when exposed to Cu and Zn.
However, among the tested heavy metals $HgCl_2$ was found to be the potent inhibitor of invertase activity. The results of the present investigation are in harmony with the results of the earlier workers (Lomte and Patil 1985, 1988; Alam 1981; Nulina Sundari, et al., 1986).

There have been comparatively less studies on the proteolytic and lipolytic enzymes of the molluscs. Extracellular protease has been found to be present in carnivorous animals rather than in herbivorous animals. c, Mathews and Murleedharan (1986) also reported a significant drop in protease activity after 24, 48 and 72 hours tropical application of Precocence – II to the caster and semilooper Achaoea Janata. Decreased digestive enzyme secretion due to sublethal doses of Methyl Parathion, affects food utilization in Spodoptera litura (Vasanta and Chocklingam, 1986).

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