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Proteotoxicity Studies in Liver, Muscle, Kidney and Gills of *Oreochromismossambicus a*nd *Labeorohita* Exposed to Plant Nutrient (Librel^{TM})

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Abstract: *Fish are one of the major sources of protein for human beings and the nutritional value of fish depends on their biochemical composition like protein, amino acids, vitamins, mineral contents, etc. The present study was done with the view to study protein composition by means of electrophoretic patterns of proteins fractions in tissues of O.mossambicus and L.rohita. The clinical value of the protein analysis by electrophoresis depends upon whether a given change represents an adaptation to stress conditions or a failure in the supportive physiological and biochemical mechanisms of the animals. In the present study there was a time dependent significant increase in the total protein content of both the fishes. This appraisal of the quantitative estimation of protein content in tissues of Librel exposed fishes indicates that apparently both species induced time dependent mild to drastic alterations as compared with the controls to face the stress cause by the exposure.*

Keywords: *Librel, proteomics, Toxicity, Fish.*

1. INTRODUCTION

Proteins occupy a unique position in the metabolism of cell because of the proteinaceous nature of all the enzymes which mediate at various metabolic pathways [1]. Therefore, the assessment of proteins can be considered as a diagnostic tool to determine the physiological process of cell [2].Proteomic analyses provide valuable information, when variations that occur within the proteome of organisms are compared as a consequence of biological perturbations or external stimuli. These stimuli often result in different protein expressions or the redistribution of specific proteins within cells [3]. We preferred to use *O.mossambicus*and *L.rohita* as the test model, mainly because these have been favored by many earlier workers as test models for both cytogenetic and molecular studies; moreover they make an appropriate model as an indicator species in biomonitoring programs [5].This study was the first to use protein profiling in a nonmodel organism (rainbow trout) to demonstrate, for the first time in teleosts, that proteomics have the potential to assist in studying cellular mechanisms involved in protein degradation. [6] Reported significant differences in fish caught from polluted and non polluted sites. An added bonus in these studies is the fact that it is not absolutely necessary to establish the identity of a protein for it to become a successful biomarker of exposure. Indeed, the characteristics of a peptide and the specific conditions under which it occurs are the more pressing concerns [7]. Moreover, the preliminary data from this study was one of the first to suggest that proteomic approaches may be useful in an environmental contamination context for studying fish, and may have potential application to serve as a high-throughput screening approach for disease classification. Such changes might reflect an altered antibody synthesis, protein biosynthesis, cellular leakage or perhaps other events resulting directly or indirectly from the stress. However, reports on variations of qualitative tissue proteins are lacking, especially with reference to Librel exposure which is micronutrient mixture containing metals (Fe, Cu, Zn, Mn and B).The genotoxicity of many agrochemicals is still under debate as there are reports which are either positive or negative; plant nutrient is one of them, for which such studies are the rare.

Exposure of Librel has led to hematological, biochemical and has resulted into serious impairment of gonads and liver tissues [8]. Further the metal accumulation is also reported in various tissues of both the teleost fish, leading to alteration in the metabolic activities, and biochemical indices of stress. So,

O.mossambicus and *L.rohita*are use as the test model, mainly because these have been favored by many earlier workers as test models for both cytogenetic and molecular studies; moreover they make an appropriate model as an indicator species in biomonitoring programs [5].The development of electrophoretic techniques makes it possible to detect the protein composition. Several studies have revealed that fish are able to accumulate and retain metals in different fish tissues [9, 10, 11, 12, 13, 14, 15] and have shown to be time dependent. Hence understanding the protein profile may add more understanding towards the outcome of the previous studies.

2. MATERIALS AND METHODS

2.1. Estimation of Total Protein Content

Total protein content was estimated by the modified method of [16].

2.2. Protein Precipitation by TCA

Before loading on SDS-PAGE, proteins were precipitated by mixing 10% TCA with tissue homogenate prepared in distilled water. Then the samples were kept in ice for 10 min and centrifuged for 10 min at 3000rpm. Again the pallet was mixed with 5% chilled TCA, kept for 10 min and centrifuged at 3000 rpm. The protein pellet was then washed with ethanol/ethyl acetate (1:1) and centrifuged for 10 mins at3000 rpm. The final protein pellets was dissolved in 0.5 N NaOH and used for SDS PAGE.

2.3. Preparation of Gel Slab

The glass plate's sandwich was assembled using two clean glass plates and 1mm Teflon spacers. The glass plates were sealed with 0.8 % agar solution. Resolving gel solution 12 % (1.5M Tris- HCl, PH 8.8 -2 ml, 30 % Acrylamide-3.2 ml, 10 % SDS-0.5 ml double distilled water-1.8 ml, TEMED-0.015 ml, Ammonium per sulfate-0.5 ml) was prepared and poured in between the clamped glass plates. To avoid entrapment of any air bubbles, the gel solution was overlaid with distilled water. The plates were left undisturbed for 30 min for polymerization of the gel. After gel polymerization, overlaid water was removed and rinsed with stacking gel buffer. Now the 5% stacking gel solution (0.5 M Tris-HCl, pH 6.8-2 ml, 30% Acrylamide-0.8 ml, 10% SDS-0.5 ml, double distilled water -1.2 ml, TEMED -0.015 ml, 1.5% Ammonium per sulfate 0.5 ml) was prepared and poured over the polymerized resolving gel, comb was inserted carefully. The gel slab was left undisturbed for 15 minutes, after polymerization comb was removed carefully

2.4. SDS- PAGE Analysis

The precipitated proteins dissolved in 0.5N NaOH was mixed in sample buffer (0.5M Tris-HCl pH-6.8-2 ml, 40% glycerol-1.6ml, 10% SDS-3.2ml, 2-mercaptoethanol-0.8ml, 0.1%(w/v) bromophenol blue-0.4 ml) at the ratio of 3:1 and heated at 60^{o}C for 10 minutes. The SDS-PAGE was performed to analyze protein profile in muscle of control and Librel exposed tissues of different time intervals by using standard method [17] the concentration of acrylamide was 12% and sample extract was loaded in each lane of the gel. The electrophoresis was carried at 100V for 2.5 hrs by watching the movement of the tracking dye and the gel was analyzed with Coomassie blue staining for visualizing protein bands.

2.5. Staining Method

The proteins separated by electrophoresis through SDS-PAGE were fixed by placing the gel in fixation solution (50:10:40 / methanol: acetic acid: H_2O) for 2 hours with gentle shaking. The fixation solution was decanted, and gel was rinsed thrice with double distilled water for 5 seconds. The gel was than stained in the staining solution for 5 minutes with gentle shaking. The developing solution was decanted and the reaction was quenched by washing the in distil water for few minutes. Then the gel was destained in destaining solution (0.1% CBB R-250/ 40% MeOH/ 10% Glacial Acetic Acid) for 5 minutes and then the gel was kept overnight for the appearance of clear bands. The electrophoretogram gel was preserved in water and then gel documented by Gel Doc (Bio Rad).

2.6. Determination of Molecular Weight of the Protein Subunits Separated on SDS PAGE

To determine the molecular weight of the individual subunits of the protein, the relative mobility of the individual subunit was calculated by using the following formula.

Relative mobility (Rm) =Distance travelled by individual subunit

Distance travelled by the marker dye

A standard curve is prepared by plotting migration distances ('X'-axis) of known protein standards against their molecular weights ('Y'-axis) on semi log. From the migration distance of an unknown protein, the molecular weight of the protein was calculated from the standard curve.

3. RESULTS AND DISCUSSION

Plant nutrient exposures lead a time dependent significant ($p<0.05$) increase in the protein content in tissues of both the fishes. The calculated values for total proteins along with standard deviation are given in Table IV. The fresh water fishes *O.mossambicus* and *L.rohita*on sub chronic exposure to low sub lethal concentration of plant nutrient revealed variation in electrophoretic protein fractions between the control and experimental fishes. As presented in the Fig. I-III electrophoretogram of both species represents an increase in the intensity of tissue protein subunits compared to control in the both the species at the initial 15th and 30th day of exposure while a decrease at the 45th day. The size of protein was extrapolated by plotting the graph of log (mol. weight) against Rm (Tables I-III).

Table I. *Time dependent changes in the protein subunits (kda) of fish muscle exposed to sub-lethal concentration of plant nutrient librel.*

Sr. No.	PM	*O. mossambicus*				*L. rohita*			
		L I	L II	L III	L IV	L I	L II	L III	L IV
1				3.46	3.46				3.46
2	3.5	3.5	3.5						
3						3.54	3.54	3.54	
4							6.30	6.30	
5		6.45	6.45	6.45					6.45
6	6.5								
7					6.6	6.60			
8							13.80	13.80	
9		14.12	14.12						14.12
10	14.3								
11				14.45	14.45	14.45			
12		19.95		19.95			19.95	19.95	
13	20.1					20.1			
14							28.18	28.18	
15		28.84							
16	29								
17				29.51		29.51			
18	43								
19						43.65	43.65	43.65	
20	66								
21	97.4								
22							100	100	
23			199.52	199.52			199.52	199.52	199.52
24	205								

PM-Protein Marker, L I – Control, L II – 15 days, L III-30 days, L IV-45 days

Polypeptides in muscle ranged from 3.46 to 199.52KDa in both species; in gills 3.46 to 97.4 in O.mossambicus and 3.56 to 43.0 in *L.rohita*; while in liver 3.54 to 43.0 in *O.mossambicus*3.46 to 63.2 in *L.rohita*. In *O.mossambicus*; muscle control showed 5 bands whereas 15th, 30th and 45th day groups showed 5, 6 and 4 bands respectively; gill control showed6 bands whereas 15th, 30th and 45th day groups showed 5 bands each while liver control showed 4whereas 15th, 30th and 45th days groups showed 5, 5 and 4 bands respectively; In *L.rohita,* muscle control showed 6 bands, whereas 9, 9 and 4 bands were seen in 15th, 30th and 45th day groups; gill control showed 5 bands whereas 10, 8 and 8 bands were seen in 15th, 30th and 45th day; while liver control showed 3 bands whereas 4, 6 and 5 bands were seen in 15th, 30th and 45th day. In *O.mossambicus*; muscle showed depletion of 28.84 and addition of new bands (3.46, 6.6, 14.45 and 29.51); gill showed depletion of (3.54, 40.0, 59.6 and 97.4) and addition of (3.46, 42.3 and 55.3) bands while liver showed while liver showed depletion of (29.1) and addition of (20.1, 22.3, 29.1) bands. In *L.rohita* muscle showed depletion of (6.60, 14.45, 20.1 and 24.51) and addition of new bands (3.46, 6.30, 6.45, 13.80, 14.12, 19.95, 28.18); gill showed

depletion of (3.54, 14.6, 20.1 and 22.3) and addition of (3.46, 4.2, 6.7, 9.1, 12.2, 14.3, 17.2 and 43.0) bands while liver showed depletion of 29.1 and addition of (3.46, 3.54, 6.5, 21.9, 22.3 and 63.2) bands.

Table II. *Time dependent changes in the protein subunits (kda) of fishgill exposed to sublethal concentration of plant nutrient librel.*

Sr. No.	PM	*O.mossambicus*				*L.rohita*			
		L I	L II	L III	L IV	L I	L II	L III	L IV
1			3.46	3.46	3.46		3.46	3.46	3.46
2	3.5								
3		3.54				3.54			
4							4.2		4.2
5	6.5								
6							6.7	6.7	6.7
7		8.2	8.2	8.2	8.2	8.2	8.2	8.2	
8							9.1		
9							12.2	12.2	12.2
10	14.3						14.3	14.3	14.3
11						14.6			
12							17.2	17.2	17.2
13	20.1	20.1	20.1	20.1	20.1	20.1			
14						22.3			
15							17.2	17.2	17.2
16	29								
17		40.0							
18			42.3	42.3	42.3				
19	43						43.0	43.0	43.0
20			55.3	55.3	55.3				
21		59.6							
22	66								
23	97.4	97.4							
24	205								

PM-Protein Marker, L I – Control, L II – 15 days, L III-30 days, L IV-45 days

Table III. *Time dependent changes in the protein subunits (kda) of fishliver exposed to sublethal concentration of plant nutrient librel.*

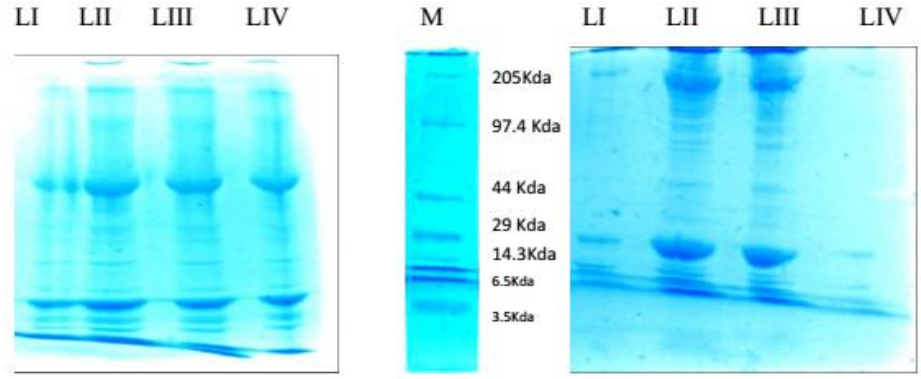
Sr. No.	PM	*O. mossambicus*				*L.rohita*			
		L I	L II	L III	L IV	L I	L II	L III	L IV
1								3.46	3.46
2	3.5								
		3.54	3.54		3.54		3.54	3.54	
3	6.5						6.5	6.5	6.5
4		14.2	14.2	14.2	14.2	14.2	14.2	14.2	14.2
5	14.3								
6	20.1			20.1		20.1			20.1
7		21.9					21.9		
8			22.3	22.3				22.3	22.3
9	29								
10			29.1	29.1		29.1			
11	43	43.0			43.0				
12								63.2	
13	66								
14	97.4								
15	205								

PM-Protein Marker, L I – Control, L II – 15 days, L III-30 days, L IV-45 days

Table IV. *Changes in protein values (mg protein/100 mg wet weight of the tissue) of fish*

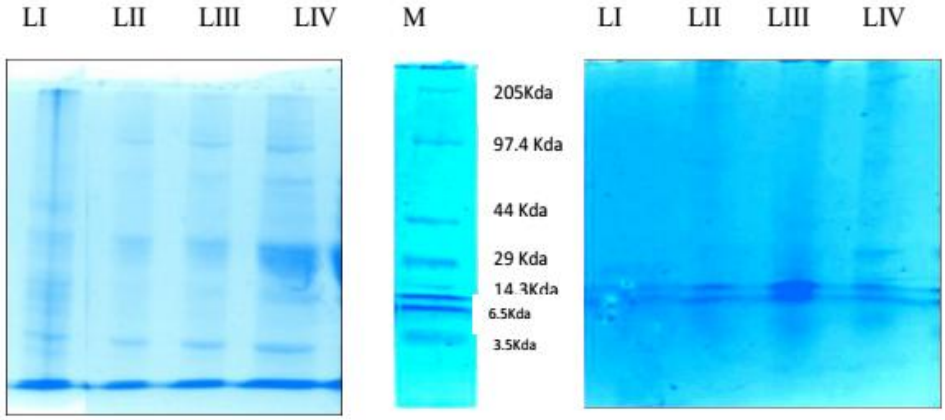
Sr. No.	*O.mossambicus*				*L.rohita*			
	Control	**15 days**	**30 days**	**45 days**	**Control**	**15 days**	**30 days**	**45 days**
Muscle	2.33 ± 0.1	5.8* ± 0.3	15.9 ± 0.5*	17.3± 0.23*	3.4± 0.78	7.3± 0.84*	20.4± 0.25*	22.2± 0.2**
Gill	5.25± 0.2	8.55± 0.08	9.11± 0.08*	16.23± 0.08	8.82 ± 0.8*	12.3± 0.25	19.22± 0.2*	32.86± 0.12
Liver	6.22± 0.7	15.2± 0.3*	19.22± 0.5	25.66± 0.08	12.1 ± 0.9*	15.2± 0.08	20.3 ± 0.2*	37.1 ± 0.3*

** The mean difference is significant at the 0.05 level; ** the mean difference is significant at the 0.01 lev*



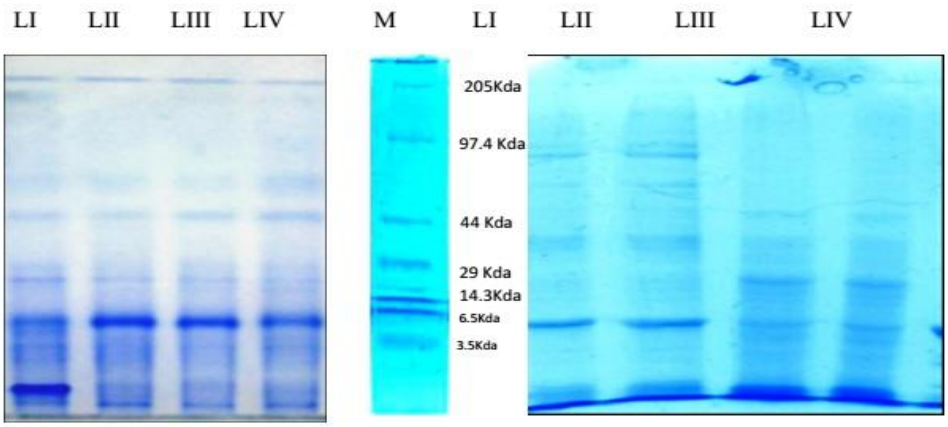
LI – Control; LII – 15 days; LIII – 30 days; LIV – 45 days

Fig1. *Electro photogram showing in protein subunits of fish muscle exposed to plant nutrient Liberal*



LI – Control; LII – 15 days; LIII – 30 days; LIV – 45 days

Fig2. *Electro photogram showing in protein subunits of fish Liver exposed to plant nutrient Liberal*



LI – Control; LII – 15 days; LIII – 30 days; LIV – 45 days

Fig3. *Electro photogram showing in protein subunits of fish muscle exposed to plant nutrient Liberal*

The present study was done with the view to study protein composition by means of electrophoretic patterns of proteins fractions in tissues of *O.mossambicus* and *L.rohita*. The clinical value of the protein analysis by electrophoresis depends upon whether a given change represents an adaptation to stress conditions or a failure in the supportive physiological and biochemical mechanisms of the animals [18]. In the present study there was a time dependent significant increase in the total protein content of both the fishes. This appraisal of the quantitative estimation of protein content in tissues of Librel exposed fishes indicates that apparently both species induced time dependent mild to drastic alterations as compared with the controls to face the stress cause by the exposure.

Further there was a species specific difference in the protein fractions when SDS-PAGE was performed. The electrophoretic profile of both the species revealed an increase in the intensity of bands upto 30 days while a decrease at 45^{th} day. This initial increase in the intensity and then a decrease could be attributed to the activation of certain proteins and then a sudden deactivation. Moreover according to [19] the increase in the band intensity suggests that such responses possibly represent a common reaction to heavy metals.

It is suggested that the occurrence of new protein in the muscle has been transported through blood stream or they could have been synthesized in the muscle itself. It is inferred that these protein fractions could be stress proteins to overcome the toxic effect of heavy metal [20]. Hence in the present study the appearance of new protein fractions in muscle could be stress proteins to overcome the toxic effect of Librel.

The expression of low molecular proteins in the experimental groups was a curious observation in all the tissues of both the species which was highest in liver. The agrochemicals may inhibit the expression of some genes (or) activate the others to produce specific mRNAs which may subsequently be translated into specific proteins called stress induced proteins [21, 22). The present study may provide an insight in rate of turnover of various proteins alterations at cellular and sub cellular levels and changes in the biological properties of fish in reference to plant nutrient. Our study has lend the findings of induction of species specific new protein bands upon exposure of trace element mixture, Hence one possible mechanism is that, when fish is trying to adjust in *in vitro* environment small fluctuations of protein band is seen, while on the hand it is also trying to combat the stress which is being produced/given. So, these metal ions which are resent in high amount is accumulated in respective tissue which lead to activation/expression of certain stress genes which are either not produced or are at low level in normal condition.

4. CONCLUSION

The study concludes that, trace metal ion poses a higher risk to the animal and disrupts the overall physiology of the both the fish which is seen in the expression pattern of the some protein which are not present in control condition. However, western blot studies will throw more light on the specific protein expression to unveil the mechanism of action of this plant nutrient mixture.

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