



Thermodynamic Characterization of Kidney Superoxide Dismutase from *Labeo rohita* Exposed to the Mixture of Lead and Chromium

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ABSTRACT

Toxic effect of lead (Pb)+chromium (Cr) mixture on superoxide dismutase (SOD) from kidney of *Labeo rohita* exposed to sub-lethal concentration for 14 days was evaluated. After purification, specific activity of enzyme was 1750.13 and 1272.72 U mg⁻¹ in kidney of metal exposed and control fish, respectively. The enzyme from metal exposed kidney was 2.68 fold purified with 58.73 % age recovery. Results of characterization showed that SOD had broad range of pH from 4 to 8.5 but show maximum activity at pH 7 and 7.5 for stressed and control fish, respectively. At 30 °C activity of SOD was maximum for both control and stressed fish. At 40 °C the enthalpy of denaturation (ΔH^*) for metal treated and control fish were 0.373 and 0.736 KJ mol⁻¹, respectively and decreases with further increase in temperature (70 °C) until it was 0.132 and 0.495 KJ mol⁻¹, respectively. The free energy (ΔG^*) of thermal denaturation for metal treated and control fish were 58.45 and 58.79 KJ mol⁻¹, respectively at 40 °C and increases with increase in temperature until it remained 62.65 and 63.18 KJ mol⁻¹, respectively at 70 °C. The negative value of entropy of inactivation (ΔS^*) shows that the superoxide dismutase is stable thermodynamically.

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Authors' Contribution

HN executed this research. SA planned the research. KA helped in writing article. AZ guided the author in planning the research work.

Key words

Antioxidant enzyme, Chronic exposure, Fish, Heavy metals mixture.

INTRODUCTION

Inorganic chemicals such as heavy metals are most important pollutants of aquatic ecosystem worldwide. Major sources of metal pollution are mining, industry, advanced agriculture, motor traffics and household waste. Pollution from these resources has increased day by day with the technological progress of human society. These metals can persist in water and sediments, and have ability to accumulate in aquatic organisms especially in fish (Luoma and Rainbow, 2008).

Fish capture a place at the apex of food chain with greater likeliness of accumulating toxicants from the aquatic environment. Metals are able to induce oxidative stress and therefore can be used as a potential biomarker for assessing the oxidative damage and metal pollution in aquatic ecosystem (Livingstone, 2003). Chronic exposure of heavy metals for long period does not cause mortality but have adverse toxic effect on organism (Kumara *et al.*, 2010). Both lead and chromium have toxic effects on fish tissues, like enzymatic changes due to oxidative stress (Patil and David, 2013).

Like the other animals, the fish as well possess an anti-oxidant resistance mechanism to deter the negative

impacts of reactive oxygen species (ROS). The antioxidant resistance system of fish is dependent on many factors. These factor may be internal or external and either weaken or fortify the antioxidant resistance system of the fish (Martinez-Alvarez *et al.*, 2005). Oxidative stress can result from a number of factors, however, it is directly correlated with ROS production (Finkel and Holbrook, 2000; Hensley and Floyd, 2002). The construction of reactive oxygen species and reactive oxygen intermediates is an inevitable result in the bulk of take breaths living creatures (Kim *et al.*, 2007). The antioxidant defense system of fish including enzymes superoxide dismutase, glutathione, metallothioneins and catalase activated after the production of ROS (Bagnyukova *et al.*, 2006). One of the parental forms of intercellular reactive oxygen species is O⁻², it can be transformed into hydrogen peroxide and oxygen by superoxide dismutase. Evaluation of toxic effect of metals on antioxidant enzymes is a useful tool in toxicological research (Velma and Tchounwou, 2010). Keeping in view the toxicity of lead and chromium to environment and fish, we planned to study the sublethal effect of Pb+Cr mixture on the superoxide dismutase in kidney of *Labeo rohita*.

MATERIALS AND METHODS

Fish and experimental layout

Labeo rohita, commonly known as rohu, was selected

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as an experimental animal. The experiment was conducted at Fisheries Research Farms, University of Agriculture, Faisalabad, Pakistan. The fish were acclimatization to laboratory conditions for 14 days. During acclimatization 12 h light and 12 h dark photoperiod was maintained. Chemically extra pure chloride salts of lead and chromium were used to prepare stock solutions of desired dilution. After two week, fish was transferred to 35-liter glass aquaria for oxidative stress studies. Ten fish were kept in each aquarium. Sublethal (1/3 LC₅₀) exposure of Pb+Cr mixture (1:1 ratio) was given to *L. rohita* for 14 days. The 96 h LC₅₀ value of Pb+Cr mixture was 33.11mgL⁻¹ as determined by [Batool and Javed \(2015\)](#). The sub-lethal value of Pb+Cr mixture for *L. rohita* was 11.1 mg L⁻¹. Along this a control group was also run without metal mixture exposure. At the end of experiment, the fish were dissected and kidney was separated from both control and Pb+Cr mixture stressed *Labeo rohita* and stored for further biochemical analyses at -20°C.

Extraction and purification of SOD

Dissected organ *i.e.* kidney was homogenized in cold phosphate buffer (0.2 M, pH 6.5) by the ration of 1:4 w/v. Homogenate organ was centrifuged at 10,000 rpm for 15 min at 4°C. After this, clear supernatant was separated for enzyme assay while residue was discarded. The activity of superoxide dismutase was determined by following the method of [Giannopolitis and Ries \(1977\)](#). Crude enzyme was purified by ammonium sulphate precipitation using the method of [Crapo *et al.* \(1978\)](#). Partially purified SOD was further purified by ion exchange chromatography by following the method of [Zia *et al.* \(2007\)](#). For purification of SOD, column of DEAE-cellulose (diethyl amino ethylcellulose) was prepared and 0.25ml of desalted enzyme sample was applied on column. The sample was eluted out with the help of 0.067 mM phosphate buffer (pH 7.8) while the drop rate was kept constant (1 mLmin⁻¹). A total of 50 fractions with 2 mL of elution were collected. The optical density of all the fractions was noted at A₅₆₀ nm against blank (buffer). Fractions having higher absorbance were chosen for enzyme assay and total protein content estimation.

The protein content in kidney tissue was measured by following Biuret method ([Gornall *et al.*, 1949](#)) using bovine serum albumin (BSA) as a standard.

Kinetic characterization and thermodynamic study

Determination of kinetic parameters

Optimum pH was determined by assaying the superoxide dismutase at different pH ranging from 4-8.5 with each 0.5 difference. To get the optimum temperature for the enzyme, it was assayed at different temperatures (20, 30, 40, 50, 60, 70 and 80 °C) keeping the pH 7.0 for

control and 7.5 for stressed *L. rohita*. Activation energy was determined by assaying the superoxide dismutase at different temperatures ranging from 0-80 °C. Data obtained was plotted in Arrhenius plot. For this purpose method described by [Obedunmi and Owalude, \(2007\)](#) was employed. The mechalis-menton kinetics contant (K_m and V_{max}) was determined by assaying the different concentrations of NBT (0, 0.5, 1.0, 1.5, 2.0, 2.5 mM) as described by [Leiter *et al.* \(2004\)](#).

Determination of thermodynamic parameters

For the determination of thermal denaturation enzyme was incubated at 40-80°C for 15 min in an incubator with the same amount of phosphate buffer of pH 7.0 for controlled and 7.5 for stressed *L. rohita*, afterward was cooled in ice for 30 min and assayed for activity. Data obtained was plotted in first order plot, rate constants for thermal denaturation (K_d) were determined and Arrhenius plot was applied to determine the activation energy for denaturation ([Zia *et al.*, 2007](#)). The thermodynamic parameters for thermostability were calculated by rearranging the Eyring's absolute rate equation derived from the transition state theory ([Eyring and Stearn, 1939](#)).

$$K_d = (k_b/h) e^{(\Delta H^*/RT)} \cdot e^{(\Delta S^*/R)} \quad (a)$$

$$\Delta H^* = E_a^* - RT \quad (b)$$

$$\Delta G^* = -RT \ln \{K_d (h/ K_b, T)\} \quad (c)$$

$$\Delta S^* = (\Delta H^* - \Delta G^*)/T \quad (d)$$

Where, H is Planck's constant (6.63 x10⁻³⁴ Js), K_b is Boltzman's constant (R/N) (1.38 x 10⁻²³ JK⁻¹), R is Gas constant (8.314 JK⁻¹mol⁻¹), N is Avogadro's No. (6.02 x10²³ ml⁻¹), T is absolute temperature, ΔH* is enthalpy of activation of denaturation, E_a* is activation energy for denaturation, ΔG* is free energy for denaturation, and ΔS* is entropy of activation of denaturation

Statistical analyses

Data obtained was analyzed by appropriate methods of Statistics ([Steel *et al.*, 1996](#)). MS Excel and Slide write plus software were used to draw graphs. Two-way ANOVA was used to compare variables among both metal stressed and control fish at *p*<0.05.

RESULTS

Ammonium sulphate precipitation

In the present study partially purified enzyme from kidney of control fish *L. rohita*, had attained 1.75 fold purification and 60.65% recovery as compared to the crude enzyme. However stressed fish had attained 1.43 fold purification and 61.33% recovery in kidney. It was noticed that the activity of SOD in kidney of stressed fish was higher as compared to control. Results indicated that the activity of SOD was decreased and specific activity

Table I.- Purification summary of SOD in kidney of *L. rohita*.

Precipitation steps	Treatments	Activity (U _{mL} ⁻¹)	Protein (mg mL ⁻¹)	Specific activity (U mg ⁻¹)	Fold purification	% age yield
Crude	Control	554.54	1.10	504.12	1	100
	Stressed	681.81	1.05	648.57	1	100
Salting in 60%	Control	527.27	0.68	775.39	1.53	95.08
	Stressed	563.63	0.72	782.81	1.20	82.66
Salting out 80%	Control	436.36	0.52	839.15	1.66	78.68
	Stressed	527.27	0.62	850.43	1.31	77.33
Desalting	Control	336.36	0.38	885.15	1.75	60.65
	Stressed	418.18	0.45	929.28	1.43	61.33
DEAE-Cellulose	Control	318.18	0.25	1272.72	2.52	72.13
	Stressed	400.00	0.23	1739.13	2.68	58.73

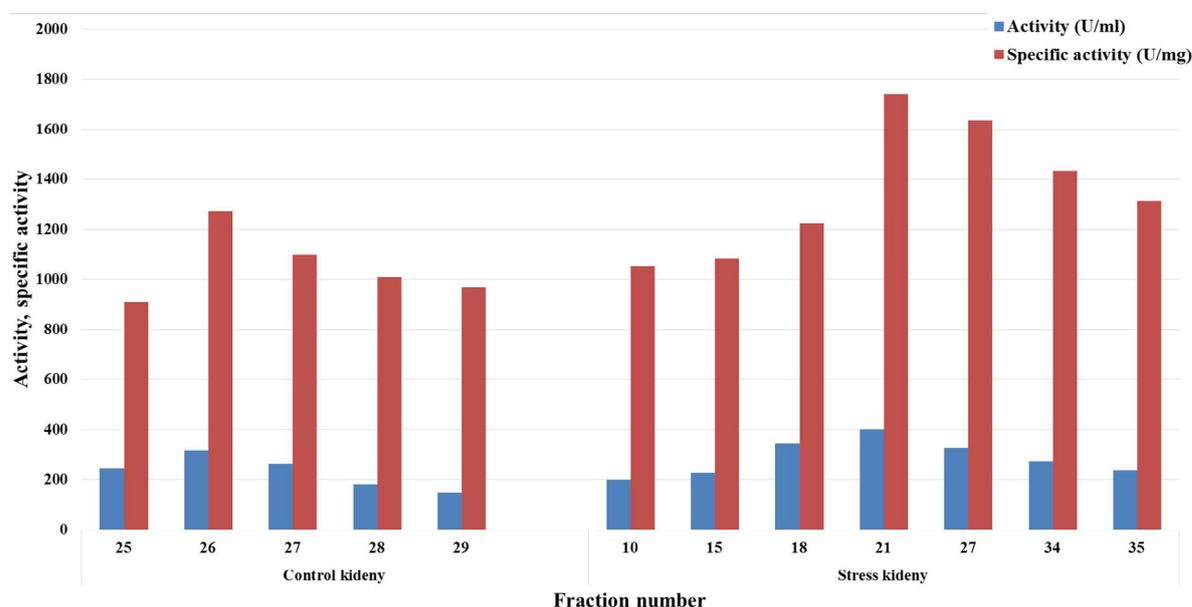


Fig. 1. Fractional summary of SOD by using ion-exchange column chromatography.

was increased after each step of purification (Table I). The percentage increase/decrease in SOD activity and specific activity is given in Table II.

Ion exchange chromatography

It was found that 26th fraction from kidney of control fish had the highest enzyme activity of 318.18 U_{mL}⁻¹, specific activity of 1272.72 U_{mg}⁻¹ and enzyme was 2.52 fold purified and %age recovery was 72.13. While 21th fraction from kidney of stressed fish had highest activity of 400 U_{mL}⁻¹, specific activity of 1739.13 U_{mg}⁻¹ and enzyme was 2.68 fold purified and %age recovery was 58.73 (Table I; Fig. 1). Results showed that the activity and specific activity of superoxide dismutase at concentration of 11.1 mg L⁻¹ (Pb+Cr) accelerated by 25.72 and 36.65%, respectively in kidney of *L. rohita* when compared with

control (Table II). All results were highly significant statistically at p<0.01.

Kinetic and thermodynamic characterization

Optimum pH

Enzymes showed highest activity at optimum pH. In present study results indicated that the enzyme showed a wide range of pH from 4.0-8.5. It was observed that enzyme from kidney of control and stressed *L. rohita* showed maximum activity at pH 7.0 and 7.5, respectively (Fig. 2A).

Effect of temperature

The activity of the enzyme was changed when treated with temperature, so on the basis of temperature the enzyme superoxide dismutase was observed for stability changes.

At 30 °C activity of superoxide dismutase was maximum for both untreated and stressed *L. rohita* when range was 20 to 80 °C. Figure 2B shows a declined in activity with increase in temperature.

Table II.- Percentage increase/decrease over control for SOD from kidney of *L. rohita*.

Precipitation steps	Activity (U mL ⁻¹)	Specific activity (U mg ⁻¹)
Crude	22.95	28.65
Salting in 60%	6.90	0.96
Salting out 80%	20.83	1.22
Desalting	24.33	4.99
DEAE-Cellulose	25.72	36.65

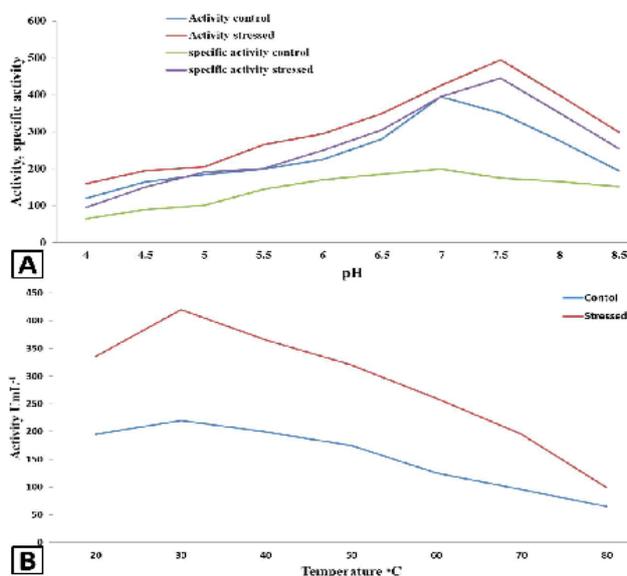


Fig. 2. Effect of different pH (A) and temperature (B) on SOD activity.

Effect of substrate

The SOD exhibited low value of K_m for kidney of *L. rohita* which shows that the SOD had high affinity for NBT. The K_m value for kidney of control and stressed *L. rohita* was calculated as 0.190 and 0.59 mM, respectively. The V_{max} for Kidney SOD of control and stressed fish was estimated as 1025 and 595 U mL⁻¹, respectively (Figs. 3, 4B).

Thermodynamics of irreversible thermal inactivation

Thermostability is the ability of enzymes to resist against thermal unfolding at elevated temperatures in the absence of substrate. Arrhenius plot for the determination

of energy of activation (E_a) for irreversible thermal inactivation of SOD was used. It is obvious from the results that the half-life of SOD from control (Fig. 4A) and

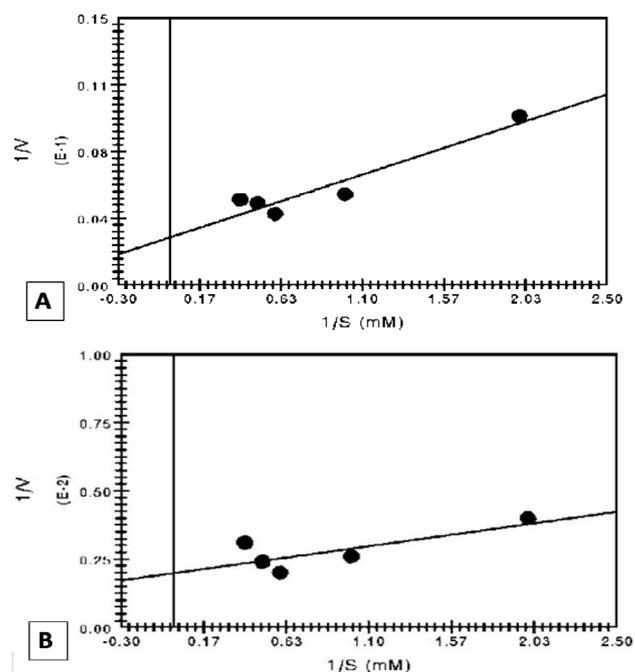


Fig. 3. Effect of NBT concentration on SOD activity in the kidney of control (A) and stressed (B) *L. rohita*.

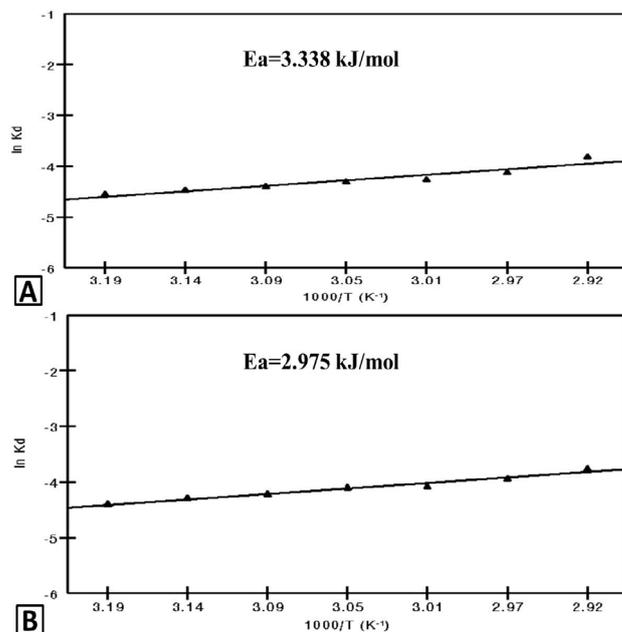


Fig. 4. Arrhenius plot for the determination of irreversible thermal denaturation of SOD from kidney of control (A) and Pb+Cr exposed (B) *L. rohita*.

stressed kidney (Fig. 4B) was 64.76 and 55.88 min, respectively at 40 °C and decreases with increase in temperature until it remains 48.80 and 40.52 min at 70°C (Fig. 4). As results of thermal inactivation of superoxide dismutase in kidney of *L. rohita* was treated at different temperatures. The results demonstrated that the enthalpy of denaturation (ΔH^*) for kidney of controlled (Fig. 5A) and stressed (Fig. 5B) *L. rohita* was 0.736 and 0.373 KJ mol⁻¹, respectively at 40 °C and decreases with increase in temperature until it remains 0.495 and 0.132 KJmol⁻¹, respectively at 70 °C (Fig. 5). The free energy (ΔG^*) of thermal denaturation for kidney of controlled and stressed *L. rohita* was 58.79 and 58.45 KJmol⁻¹, respectively at 40 °C and increased with increase in temperature until it remains 63.18 and 62.65 KJmol⁻¹, respectively at 70°C. With increasing temperature slight increase in the value of free energy was observed until at 75°C which indicating resistance of superoxide dismutase against higher temperatures. The negative value of entropy of inactivation (ΔS^*) shows that the enzyme superoxide dismutase is stable thermodynamically. Data related to this study is given in Table III.

DISCUSSION

In oxidation of biological macromolecules transition metals act as a catalyst and their toxicity may depend upon the damaged organ. Both redox active metals (Cu, Fe and Cr) and redox-inactive metals (Pb, Hg and Cd) can induced production of reactive oxygen species which cause oxidative stress leading to oxidation of lipid, proteins and DNA (Pinto *et al.*, 2003). Fish organs like liver and kidney have antioxidant defense systems to save them from oxidative stress caused by toxicants (Atli *et al.*, 2006) and useful tool to detect the chronic effect of metals (Atli and Canli, 2010). Kidney is essential organ for the elimination of environmental toxicants. Kidney evolved various mechanisms for absorbance and excretion of these toxicants (Arreola-Mendoza *et al.*, 2011; Gonzalez *et al.*, 2011). Superoxide dismutase belongs to the family of

antioxidant enzyme that converts the superoxide into H₂O₂ and O₂ (Lin *et al.*, 2008).

Present research work was carried out to evaluate the effect of Pb+Cr mixture on SOD enzyme in kidney of *L. rohita*. Results showed that the activity of superoxide dismutase accelerated by 25.72% in kidney of *L. rohita* at sublethal concentration (11.1 mg L⁻¹) of heavy metals mixture when compared with control. Results were highly significant statistically at p<0.01. These results are also similar to Vinodhini and Narayanan (2009) who evaluated the toxicity of heavy metals pollution on superoxide dismutase in kidney of common carp. They demonstrated

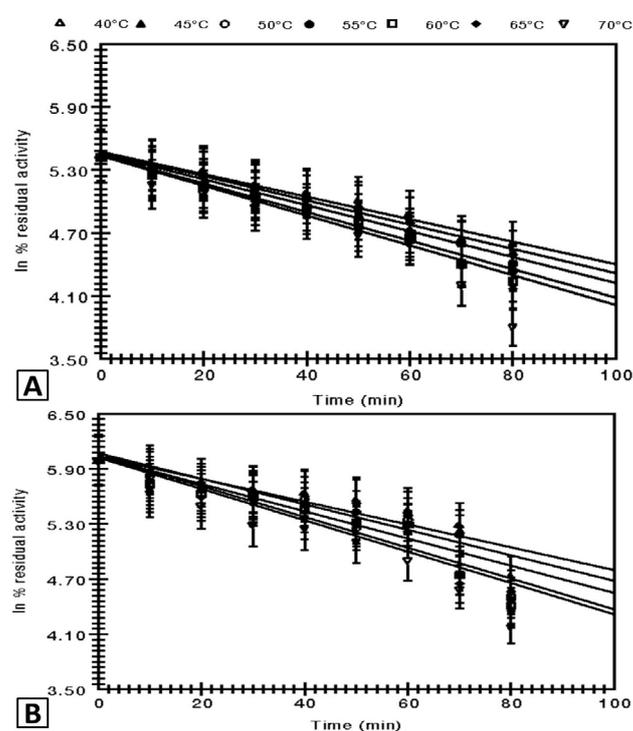


Fig. 5. Pseudo-first order plot of irreversible thermal inactivation of SOD from kidney of controlled (A) and Pb+Cr exposed (B) *L. rohita*.

Table III.- Thermodynamic parameters for irreversible thermal Inactivation of SOD in kidney of *L. rohita*.

Temperature (K)	K_d (min ⁻¹)		$T_{1/2}$ (min)		ΔH^* (KJ mol ⁻¹)		ΔG^* (KJ mol ⁻¹)		ΔS^* (JK ⁻¹ mol ⁻¹)	
	Control	Stressed	Control	Stressed	Control	Stressed	Control	Stressed	Control	Stressed
313	0.0107	0.0124	64.76	55.88	0.736	0.373	58.796	58.458	-0.185	-0.186
318	0.0115	0.0139	60.26	49.85	0.698	0.335	59.425	58.92	-0.184	-0.184
323	0.0124	0.0148	55.88	46.82	0.653	0.29	60.191	59.716	-0.184	-0.183
328	0.0136	0.0168	50.95	41.25	0.612	0.249	60.816	60.240	-0.183	-0.183
332	0.0142	0.0171	48.80	40.52	0.578	0.215	61.422	60.90	-0.183	-0.182
373	0.142	0.0171	48.80	40.52	0.537	0.174	62.293	61.77	-0.183	-0.182
342	0.0142	0.0171	48.80	40.52	0.495	0.132	63.185	62.65	-0.183	-0.182

Control, 3.338 kJ/mol; stressed, 2.975 kJ/mol calculated from Figure 4.

that under chronic stress SOD activity was accelerated in kidney after 16 days of exposure and then declined at the 32nd day. The elevated level of superoxide dismutase in kidney of common carp may be a defended system against metal toxicity. These views were also similar to [Jeziarska and Witeska \(2001\)](#) who observed that the activity of SOD enzyme in kidney may increase in response to heavy metals. The activity of superoxide dismutase was increased under exposure of pollution ([Dimitrova *et al.*, 1994](#)). The chronic exposure of heavy metals accelerated the renal antioxidant defense ([Shaik *et al.*, 1999](#)).

[Mohanty *et al.* \(2000\)](#) evaluated the cadmium toxicity to *Labeo rohita* (rohu) at different sublethal concentrations. The activity of antioxidant enzyme was enhanced under heavy metal exposure. [Basha and Rani \(2003\)](#) determined the activity of superoxide dismutase in kidney of *Oreochromis mossambicus* which are chronically exposed to heavy metal cadmium chloride (Cd²⁺) at concentration of 5ppm. Activity was increased significantly in kidney up to 15th day while reduced at the end of month. The activity of SOD was stimulated in 86.32%. Results also indicated that the antioxidant enzyme play an important role against toxic effect of Cd²⁺. [Doherty *et al.* \(2010\)](#) also reported the elevated level of SOD in different organs of fish under metal toxicity.

Our results also supported by [Farombi *et al.* \(2007\)](#) who observed the response of antioxidant enzymes in different organs of *Clarias gariepinus* under heavy metals (lead, zinc, copper, cadmium and arsenic) toxicity. Findings proved that the SOD action was improved in kidney by 50% (P <0.001). [Atli and Canli \(2010\)](#) found that the activity of SOD did not altered significantly in the kidney of *Oreochromis niloticus* exposed to sublethal concentrations of metal. Results demonstrated that response of enzyme against metal toxicity depend upon metals exposure and tissue types.

The bioassay techniques are important basis especially in the perspective of environment ([Oshode *et al.*, 2008](#)). Ammonium sulphate precipitation is very appropriate method due to the great solubility and strong ionic power of ammonium sulfate. This technique has ability to salt out the protein from the sample ([Voet *et al.*, 1999](#)). This result also supported by [Zia *et al.* \(2001\)](#) that NH₂SO₄ when added in correct quantity leads to the desired protein's precipitation and residual protein is left in the solution. Further, the pollution substances are eliminated by desalting with an increased activity.

In ion exchange chromatography column filled with resin which has charged particle. Ion exchange chromatography uses differences in signs and degree of charge of protein at given pH. The column medium is a resin having charged particles. The resemblance of each protein

for charged group is dependent upon pH and concentration of challenging free salt ions in adjacent solution ([Nelson and Cox, 2008](#)). Enzyme purification was done by using ion exchange chromatography technique after desalting process. Resin which is used in ion exchange column has ability to take up exchange able ion which is called ion exchange capacity of resin. For interchange the ion this material provides easy way to inorganic substance.

[Ken *et al.* \(2003\)](#) cloned Cu/Zn SOD from the zebra fish. They purified Cu/Zn SOD and checked the definite activity. The results proved that enzyme had definite activity 2000 U mg⁻¹. [Lin *et al.* \(2001\)](#) reported that the SOD had specific activity of 3318 U mg⁻¹ in black porgy. The results proved that the enzyme had. The pH range at which an enzyme undergoes a change in activity can provide a clue to the type of amino acid residue involved ([Nelson and Cox, 2008](#)). [Rafique *et al.* \(2018\)](#) observed the kinetic characteristics of SOD isolated from the kidney of silver carp under chronic exposure of Pb+Cr mixture. As a result of ion exchange chromatography SOD from kidney of control and Pb+Cr treated fish had highest activity as 508.33 and 427 U mL⁻¹ while highest specific activity was 1105.06 and 1055.55 U mg⁻¹ with 1.98 and 2.70 fold purification, respectively. Recovery was 70% and 68% in kidney of control and Pb+Cr treated fish, respectively. SOD showed maximum activity at pH 7.5 and 6.5 for control and treated fish kidney, respectively. At 40°C and 50°C activity of SOD was maximum for unstressed and stressed kidney of *H. molitrix* i.e. 525 and 550 U mL⁻¹, respectively. The enzyme Cu/Zn SOD from fish *Acanthopargus schligeli*, muscle was characterized. The enzyme retained maximum its activity of 24.2% and 11.3% at pH 3.0 and pH 2.2 in a stability test performed by [Lin *et al.* \(2001\)](#), so the enzyme had broad pH range from 5.8 to 11.2. [Ken *et al.* \(2003\)](#) were cloned Cu/Zn SOD from the zebra fish. They purified Cu/Zn SOD and stability of enzyme was characterized by reacting temperature. At 70 °C, enzyme was still active after heated for 10 min. [Lin *et al.* \(2001\)](#) tested the SOD activity in blackporgy, *Acanthopargus schegeli*. They purified Cu/Zn SOD from fish muscle. The results proved that at 80°C thermal inactivation rate constant of the enzyme is -0.0237 min⁻¹ and inactivation, the half-life was 27.8 min⁻¹. Total protein content decreased by 32.17% in *Labeo rohita* under sublethal metal stress ([Mohanty *et al.*, 2013](#)).

CONCLUSION

The results of this study proved that the sub-lethal exposure of lead and chromium mixture increase the SOD activity in kidney of *Labeo rohita*. These findings suggest that oxidative stress biomarkers are helpful for detection of heavy metals induced toxicity in aquatic organisms.

Statement of conflict of interest

The authors declare no conflict of interest.

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