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Thermal stability of purified superoxide dismutase from liver of commercially valuable fish, *Labeo rohita* Exposed to Pb+Cr mixture

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In this experiment, effect of lead (Pb) + chromium (Cr) mixture on superoxide dismutase (SOD) in the liver of *Labeo rohita* at a concentration of 11.1 mgL^{-1} was observed. The ammonium sulphate precipitation and ion exchange chromatography techniques were successfully used to purify SOD. After purification, SOD activity of control and Pb+Cr treated fish was noted as 581.00 and 645.45 UmL^{-1}, respectively while the specific activity was 1383.33 and 1613.62 Umg^{-1}, respectively. The fold purification value of SOD was 2.75 and 2.45 for control and stressed fish, respectively. The recovery was calculated as 77.06 and 57.43% for control and stressed fish, respectively. The results of kinetic characterization showed that SOD form control and exposed fish had maximum activity at pH 6.5 and 7.0. Temperature also had a significant effect on activity of SOD. The SOD activity was measured maximum at 30°C for both control and Pb+Cr exposed fish. The K_m value of liver SOD for control and Pb+Cr treated *L. rohita* was calculated as 1.48 and 0.62 mM, respectively. The value of V_{max} for SOD from liver of control and Pb+Cr exposed fish was 1000 and 570 U mL^{-1}, respectively. The enthalpy of denaturation (ΔH^*) for liver SOD from control and Pb+Cr exposed *L. rohita* was computed as 3.492 and 2.802 KJ mol^{-1} at 40°C, respectively and these values were dropped off with increasing the temperature until it remains 3.251 and 2.561 KJ mol^{-1} at 70°C, respectively. The free energy of thermal denaturation (ΔG^o) of liver SOD was slightly increased with increasing temperature until 75°C which shows its resistance against heat. The values of ΔG^o was observed as 58.03 and 57.95 KJ mol^{-1} for control and exposed fish at 40°C, respectively while the same was increased upto 62.37 and 62.00 KJ mol^{-1} at 70°C, respectively. It was concluded from negative value of ΔS^* (entropy of inactivation) that the SOD is stable thermodynamically.
Keywords: SOD, purification, characterization, heavy metals mixture, fish

INTRODUCTION

The pollution of freshwater bodies with a broad range of toxicants has become a hot issue all over the world. A major source of aquatic pollution includes industries, domestic and anthropogenic activities which released heavy metals in a large amount. These heavy metals may severely affect the ecological balance when they enter into the aquatic bodies (Aladesanmi, 2014). Heavy metals have ability to amass in the body of organism inhibited in aquatic bodies, which affects not only productivity and reproductive capabilities of the animals, but also human health that consume these organisms as a food source. Like, other animals, fish are an organism which cannot escape from injurious effects of these pollutants (Areola, 2007).

Lead (Pb^{+2}) is a prominent toxicant that has no important role in biological processes of life and may exert negative impacts upon long-term exposure (Mager, 2012). The mechanism of Pb induced toxicity is yet not clear, but it is evidenced that stimulate oxidative stress by forming the reactive oxygen species (ROS) (Dewanjee et al., 2013). Chromium (Cr) is also listed as a toxic and mutagenic element due to its ability to cross cell membrane and make complexes with intracellular macro-molecules such as genetic material (Venkatramreddy and Paul, 2011; Rahman et al., 2012).

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The ROS produced by heavy metals can cause the oxidation of macro-molecules like protein, lipids and RNA/DNA. Organisms have an efficient defensive mechanism of enzymes to reduce the injurious effects of ROS which includes superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and reduced glutathione (GSH), and non-enzymatic vitamin E (Trenzado et al., 2006; Jankowiak et al., 2015). Many authors conducted the research on heavy metals induced biochemical alterations in both organs and blood of different species of fish (Rani, 2000). These biochemical parameters such as antioxidant enzymes are considered to be a very sensitive biomarker to evaluate the existence of pollutants before the detrimental effects appear in aquatic individuals (Gul et al., 2004; Cazenave et al., 2014). Temperature is one of abiotic factors that is not only limits the geographical distribution of living organisms but can also affect the biological processes (Windisch et al., 2011). Fish is a poikilotherm, its metabolic rate is related to environmental temperature (Clarke and Fraser, 2004), and so temperature is considered as a stressing factor for aquatic animals (Wedemeyer et al., 1990). Studies reveal that the thermal tolerance of an organism is closely related to its aerobic ability because both heating and cooling may disturb the oxygen balance in the organs, by stimulating the production of ROS. Keeping in view above mentioned Cr and Pb toxicity to fish, this study was conducted to evaluate the sublethal effects of metals mixture on liver superoxide dismutase of *Labeo rohita*.

MATERIALS AND METHODS

***Experimental layout*:** *Labeo rohita* was used as an experimental fish. The trial was performed in Wet Laboratory at Fisheries Research Farms, UAF, Pakistan. Specimen was acclimatized to the laboratory environment for few days by maintaining the 12hr light and 12hr dark photoperiod. After 14 days, fish (n=10) were shifted to 75-liter glass aquaria for experimental trial. The stock solutions for experiment were prepared from pure chloride salts of lead (Pb) and chromium (Cr). Fish were kept under sub-lethal concentration, 11.1 mg L^{-1} of Pb+Cr mixture (1:1 ratio) calculated on the base of 96 h LC_{50} (33.11mgL^{-1}) which was computed by Batool and Javed (2015). Fish kept in clean water was used as control group. At the end of 14 days experiment, the fish were dissected and liver was collected from both control and Pb+Cr mixture treated fish for superoxide dismutase (SOD) analyses at -20^{o}C.

***Extraction of SOD*:** The liver of fish was ground in phosphate buffer (0.2 M, pH 6.5) added by the ration of 1:4 w/v. The homogenized material was centrifuged at 12,000 rpm and 4°C for 10 min. After this, as result of centrifugation, supernatant was obtained, which was used for SOD assay.

***Ammonium Sulphate Precipitation*:** The partial purification of extracted SOD was done by ammonium sulphate precipitation (Crapo et al., 1978). The precipitation was completed in two steps consist of salting in (60%) and salting out (80%). The obtained sample was desalted in phosphate buffer (5 mM) of pH 7.8 containing EDTA (0.01mM).

***Ion Exchange Chromatography*:** Partially purified SOD was completely purified by ion exchange chromatography (Zia et al., 2007). After every step of purification, the SOD activity (Giannopolitis and Ries, 1977) and total protein contents (Gornall et al., 1949) were also estimated.

***Kinetic characterization*:** The optimum pH was noted by assaying the SOD at different pH (4.0-8.5). The optimum temperature was determined by assaying the SOD at different temperatures (20-80°C) keeping the pH optimum for control and treated fish. The substrate specificity parameters viz. K_m and V_{max} found out by assaying the SOD against various NBT (0.0-2.5 mM) concentrations (Leiter et al., 2004) by Lineweaver-Burk plot.

***Thermodynamic characterization*:** The Ea for SOD was calculated from Arhennius plot (Obedunmi et al., 2006). Thermal denaturation was determined according to Zia et al. (2007). The thermodynamic parameters were calculated from the Eyring's absolute rate equation derived from the transition state theory of Eyring and Stearn (1939).

Data analysis: Following equations were used to analyze the data

(1) $Kd = (k_b/h)\, e^{(\Delta H/RT)}.e^{(\Delta S^*/R)}$

(2) $\Delta H^* = Ea^* - RT$

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

(4) $\Delta S^* = (\Delta H^* - \Delta G^*)\, /T$

Where, Kb is Boltzman's constant (R/N) 1.38 x 10^{-23} JK^{-1}, H is Planck's constant (6.63 x10^{-34} Js), N is Avogadro's No. (6.02 x1023 ml^{-1}), R is Gas constant (8.314 $JK^{-1}mol^{-1}$), ΔH* is Enthalpy of activation of denaturation, T is Absolute temperature, ΔG* is Free energy for denaturation, Ea* is the Activation energy for denaturation and ΔS* is Entopy of activation of denaturation. Graphs were drawn in MS Excel and Slide write plus software.

RESULTS

***SOD Purification*:** After purification, SOD activity of control and Pb+Cr exposed fish was noted as 581.00 and 645.45 UmL^{-1}, respectively while the specific activity was measured 1383.33 and 1613.62 U(unit) mg^{-1}, respectively. In control, 11th fraction of ion-exchange chromatography had the highest activity of SOD while in exposed fish maximum activity noted at18th fraction (Fig. 1). The fold purification value of SOD was calculated as 2.75 and 2.45 for control and stressed fish, respectively. The recovery was calculated as 77.06 and 57.43 % for control and stressed fish, respectively (Table 1). The total protein in liver of treated fish decreased in comparison to control and it was decreased at each step of purification.

Table 1. Purification of SOD from liver of *L. rohita*

Purification Steps	Activity (UmL^{-1})		Specific Activity ($U\ mg^{-1}$)		Protein ($mg\ mL^{-1}$)		Fold purification		% age yield	
	Control	Pb+Cr	Control	Pb+Cr	Control	Pb+Cr	Control	Pb+Cr	Control	Pb+Cr
Crude	754.00	809.00	502.66	657.72	1.50	1.23	1.00	1.00	100.0	100.0
SaltingIn (60%)	627.00	718.00	696.66	725.25	0.90	0.99	1.38	0.95	83.15	88.75
SaltingOut (80%)	609.09	672.00	801.43	840.00	0.76	0.80	1.59	1.32	80.78	83.06
Desalting	595.00	650.00	991.00	1015.6	0.60	0.64	1.97	1.47	78.91	80.34
DEAE-Cellulose	581.00	645.45	1383.3	1613.6	0.42	0.40	2.75	2.45	77.06	57.43

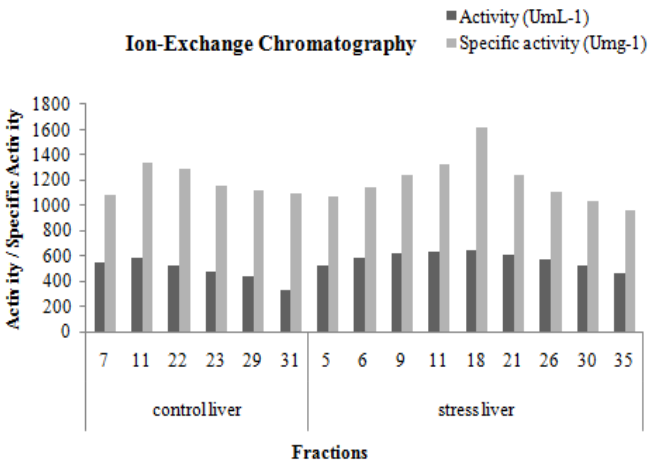

Figure 1. Fractional details of liver SOD by Ion Exchange Chromatography

Kinetic Characterization of SOD

***Optimum pH and Temperature*:** The results of kinetic characterization showed that SOD from control and exposed fish had higher activity at pH 6.5 and 7.0 (Fig. 2). Temperature also had a significant effect on activity of SOD. The SOD activity was maximum at 30°C for both control and Pb+Cr exposed fish. It was noted that SOD activity was accelerated upto a certain limit after that it was decreased (Fig. 3).

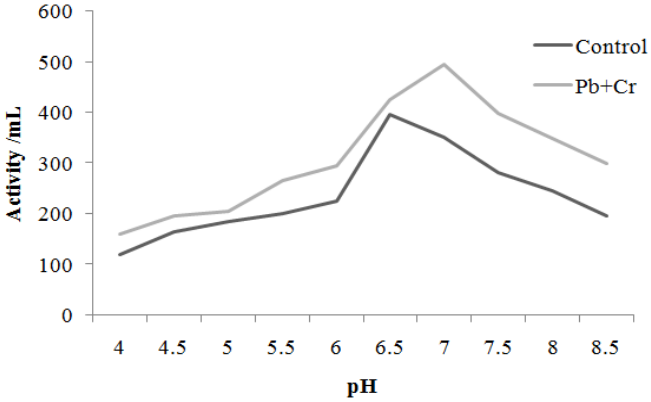

Figure 2. Alteration induced in liver SOD activity of *L. rohita* by pH

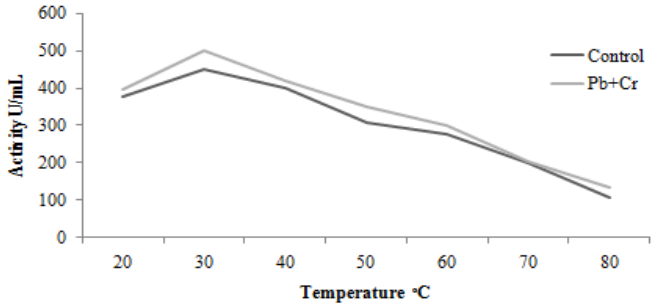

Figure 3. Alteration induced in liver SOD activity of *L. rohita* by temperature

***Substrate Specificity*:** The lower K_m value for liver SOD of fish showed its high affinity for NBT. The K_m value of SOD for the liver of control and treated fish was calculated as 1.48 and 0.62 mM, respectively. The value of V_{max} for SOD from liver of control and Pb+Cr exposed fish was 1000 and 570 U mL^{-1}, respectively (Fig. 4).

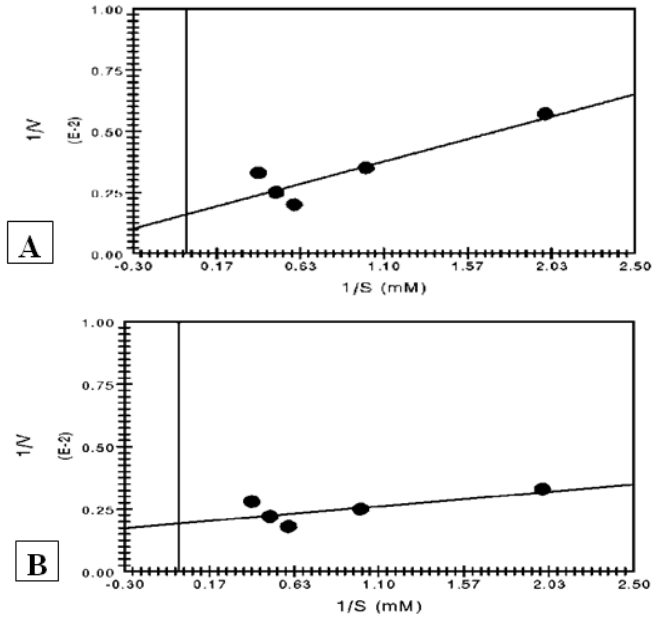

Figure 4. Impact of NBT concentration on liver SOD activity of control (A) and Pb+Cr (B) exposed *L. rohita*

Table 2. Calculations for thermal denaturation of SOD from liver of Pb+Cr exposed *L. rohita*

Temp.	K_d (min^{-1})		$T_{½}$ (min)		ΔH* (KJ mol^{-1})		ΔG* (KJ mol^{-1})		ΔS* (JK^{-1} mol^{-1})	
(K)	CL	Pb+Cr	CL	Pb+Cr	CL	Pb+Cr	CL	Pb+Cr	CL	Pb+Cr
313	0.0146	0.0154	47.79	45.00	3.492	2.802	58.033	57.958	-0.174	-0.176
318	0.0175	0.0181	39.60	38.28	3.454	2.764	58.310	58.252	-0.173	-0.174
323	0.0185	0.0186	37.45	37.25	3.409	2.719	59.117	59.102	-0.172	-0.175
328	0.0188	0.0199	36.86	34.22	3.368	2.678	59.934	59.779	-0.172	-0.174
332	0.0189	0.0215	36.66	32.32	3.334	2.644	60.633	60.277	-0.173	-0.173
337	0.0189	0.0215	36.66	32.34	3.293	2.603	61.492	61.131	-0.173	-0.174
342	0.0189	0.0215	36.66	32.34	3.251	2.561	62.372	62.006	-0.173	-0.174

Ea= Control= 6.094 kJ/mol, Pb+Cr= 5.404 kJ/mol calculated from Figure 6 and 7

***Thermodynamic Characterization*:** The activity of SOD was greatly affected by changing temperature. To see the stability changes in SOD activity against different temperature 20-80°C the activation energy for thermal denaturation (Ea) was derived from Arrhenius plot which showed a decline in SOD activity also indicates that half-life of SOD from liver of fish was also decreased with an increase in temperature (Fig. 5). The Ea for liver SOD from control and exposed fish was calculated as 6.094 and 5.404 kJ/mol.

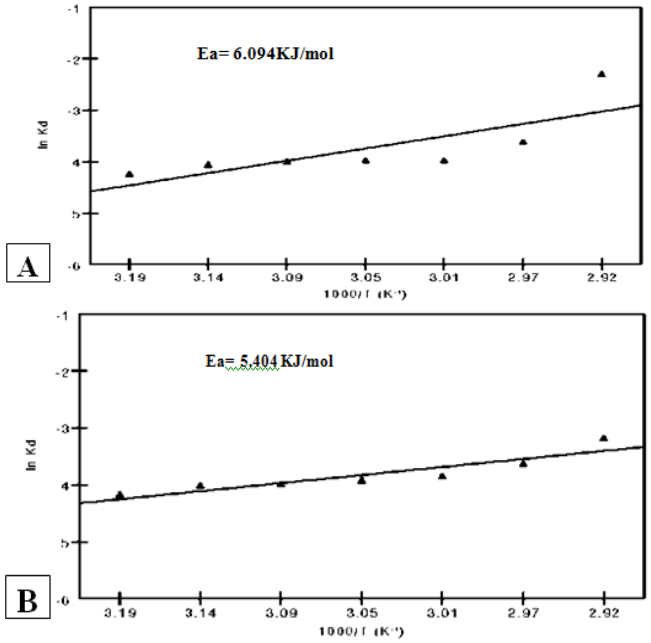

Figure 5. Arrhenius plot of irreversible thermal denaturation of liver SOD from control (A) and Pb+Cr (B) exposed *L. rohita.*

Pseduo-first order plot showed the irreversible thermal inactivation of liver SOD at different temperatures (Fig. 6). The enthalpy of denaturation (ΔH*) for liver SOD from control and Pb+Cr exposed *L. rohita* were computed as 3.492 and 2.802 KJ mol^{-1} at 40°C, respectively and these values were dropped off with increasing temperature until it remains 3.251 and 2.561KJ mol^{-1} at 70°C, respectively. The free energy of thermal denaturation (ΔG°) of liver SOD was slightly increased with increasing temperature until 75 °C which shows its resistance against heat. The values of ΔG° were observed as 58.03 and 57.95 KJ mol^{-} for control and exposed fish at 40 °C, respectively while the same was increased upto 62.37 and 62.00 KJ mol^{-1} at 70°C, respectively. It was concluded from the negative value of ΔS* (entropy of inactivation) that the SOD is stable thermodynamically (Table 2).

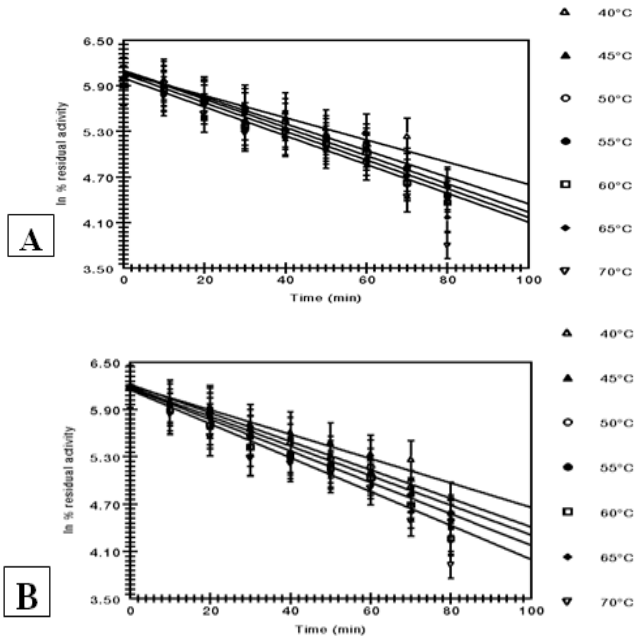

Figure 6. Pseduo-first order plot of irreversible thermal inactivation of liver SOD from control (A) and Pb+Cr (B) exposed *L. rohita*

DISCUSSION

In the present research work, Pb+Cr mixture significantly accelerated the activity of SOD in liver of *L. rohita* in comparison to control. Similarly, Velma and Tchounwon (2010) determined the chromium-induced stimulation in liver of *Carassius auratus*. Hexavalent chromium significantly

increases the SOD activity in liver of *L. rohita* under laboratory condition (Kumari et al., 2014). The SOD level was accelerated upto 61% in liver of *Clarias gariepinus* under metals stress (Farombi et al., 2007). According to Basha and Rani (2003) the SOD level in liver of *Oreochromis mossambicus* was increased significantly under sub-lethal dose of cadmium chloride. Messaoudi et al. (2009) noted the accelerated level of SOD in liver of *Salaria basilisca* exposed to cadmium for 2 and 4 weeks.

In the present research, the specific activity of SOD increased at every step of purification. Similarly, Naz et al. (2019) also noted the increased specific activity at every step. The specific activity for control and Pb+Cr exposed fish was calculated as1750.13 and 1272.72 Umg^{-1} in the kidney of *L. rohita*, respectively. Rafique et al. (2018) calculated the purified SOD activity as 508.33 and 427 UmL^{-1} from kidney of control and Pb+Cr treated fish while the specific activity was noted as 1105.06 and 1055.55 Umg^{-1}, respectively. Ken et al. (2003) also calculated the specific activity of purified Cu/Zn SOD as 2000 Umg^{-1} from zebra fish. The purified Cu/ZnSOD from the muscle of black porgy had a specific activity of 3318 U mg^{-1} (Lin et al., 2001).

In this work, the fold purification value of SOD was calculated as 2.75 and 2.45 for control and stressed fish, respectively. The yield recovery was calculated as 77.06 and 57.43 % for control and stressed fish, respectively. Naz et al. (2019) reported that the kidney SOD from control and Pb+Cr treated *L. rohita* was 2.52 and 2.68-fold purified with %age recovery of 72.13 and 58.73, respectively. Rafique et al. (2018) noted the fold purification of SOD as 1.98 and 2.70 with 70% and 68% recovery for control and Pb+Cr treated fish, respectively.

In present work, kinetic characterization results showed that SOD form control and exposed fish had maximum activity at pH 6.5 and 7.0. According to Rafique et al. (2018) at pH 7.0 and 7.5 maximum activity of SOD of control and stress fish was observed while the temperature was 40°C. The purified Cu/Zn SOD was thermally stable at 70°C when heated for 10 mints (Ken et al., 2003). In thermal stability test, the higher SOD activity from blackporgy was noted at pH 2.0 and 3.0 as 11.3 and 24.2% when the range was 5.8-11.2 (Lin *et al*., 2001). Lin et al. (2001) also calculated the thermal inactivation constant rate of SOD from black porgy was -0.0237 min^{-1} and half-life was 27.8 min^{-1} at 80°C. Naz et al. (2019) noted the maximum activity of SOD at pH 7.5 and 30 °C from the kidney of Pb+Cr exposed *L. rohita.* The thermal inactivation rate of SOD was 0.0171 min^{-1} and half-life was 40.52 min^{-1}. The total protein in liver of exposed *L. rohita* decreased as compared to control and it was also decreased at every step of purification. Mohanty et al. (2013) reported the 32.17% reduction in total protein contents of *L. rohita* under chronic metal's stress. Significantly lower protein contents in liver of fish under toxicants exposure was also noted by Tufail et al. (2019).

***Conclusion*:** In this research, exposure of Pb+Cr mixture triggers the induction of reactive oxygen species and cause acceleration in SOD activity of *Labeo rohita*. An increased level of SOD plays a crucial role in the toxicity of metals. The finding of this research also emphasizes that the biochemical parameters are useful biomarker for understanding the heavy metals toxicity in aquatic ecosystem. Moreover, ammonium sulphate precipitation and column chromatography are very simple and convenient techniques to purify the SOD.

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