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Purification and Partial Characterization of Superoxide Dismutase from Kidney of *Hypophthalmichthys molitrix* under Exposure of Metals Mixture

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Authors' Contribution
AR executed this research. HN assisted in lab work. SA guided in planning the research. KA facilitated in conducting the research work in his lab. WH helped in compiling data and statistical analysis.

Key words
Silver carp, Metal mixture, Catalase, Reactive oxygen species, Antioxidant enzyme, Superoxide dismutase, glutathione peroxidase.

ABSTRACT

In this research work Superoxide dismutase (SOD) enzyme from the kidney of silver carp (*Hypophthalmichthys molitrix*) was studied. Fish was divided into two groups. One group kept under chronic exposure of metals mixture (Pb+Cr) for 15 days and other group of fish was kept under controlled conditions. Partial purification of SOD was done by Ammonium sulphate precipitation. Ion-exchange column chromatography was used to purify enzyme for further partial characterization against different range of temperature and pH. As a result of ion exchange chromatography enzyme from kidney of control and Pb+Cr treated fish had highest activity as 508.33 and 427 UmL^{-1} while highest specific activity was 1105.06 and 1055.55 Umg^{-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. After characterization it was observed that SOD had wide range of pH *i.e.* 4.0-8.5 and maximum activity of 517 and 570 UmL^{-1} for control and treated fish kidney at pH 7.5 and 6.5, respectively. It was also observed that activity of enzyme increase with increase in temperature but upto a certain limit. At 40°C and 50°C activity of SOD was increased for unstressed and stressed kidney of *H. molitrix i.e.* 525 and 550 UmL^{-1}, respectively. At $p<0.01$ all results were statistically significant.

INTRODUCTION

From last few years, metals became largely studied in the field of toxicology as a source of environmental toxicants. Aquatic ecosystems are affected by metals (copper, cadmium, zinc, chromium and mercury) that enter in water bodies by means of agricultural, industrial and anthropogenic sources (Sampaio *et al*., 2008). Water bodies are most susceptible to metal toxicity. Toxicants, either in single and in combine form chronically affect the organism at cellular and organ level (Adeyemo *et al*., 2008). Toxicants, like metals also affect the genetic, physiology and behavior of aquatic animals (Scott *et al*., 2003). Among all the aquatic animals, fish can easily influenced by metal pollutants (Alinnor, 2005). Fish are intensively used to assess the health of aquatic systems and can be used as a bioindicator of aquatic pollution (Dautremepuits *et al*., 2004). High level of heavy metals in organs of fish are able to induce oxidative stress by producing reactive oxygen species (ROS) (Sevcikova *et al*., 2011).

Lead is very important toxicant because it has no beneficial role in development of life and produce lethal effects under long term contact (Mager, 2012). Lead enter in to the body, bind with red blood cells and move to the organs like kidney, liver, muscles, brain, heart and spleen through blood and lastly accumulate in teeth and bones (Meyer *et al*., 2008). It may cause of many diseases like kidney failure, anemia and even mortality (Yao *et al*., 2013). In gills, kidney and intestinal tissues, Cr inhibits ion-transporting ATPases (Thaker *et al*., 1996). It has been also reported that lead and chromium have ability to alter the antioxidant enzyme by producing reactive oxygen species (ROS) (Velma *et al*., 2011; Dewanjee *et al*., 2013).

Aerobic organisms have developed antioxidant defense mechanisms include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione S-transferase enzyme that scavenge ROS (Valavanidis *et al*., 2006; Droge, 2002). Elevated level of ROS adversely affects the cellular biochemistry by inducing the oxidation of lipids, proteins and nucleic acid (Livingstone, 2003). Hazardous effects of oxygen radicals are prevented by SOD enzyme which converts the superoxide radicals into H_2O_2 and O_2 (Tilly and Tilly, 1995). In field of aquatic toxicology, antioxidant enzymes

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associated with oxidative stress have become intensively studied and are used as a most responsive biomarker of aquatic pollution before any harmful effect appear in fish (Geoffroy *et al*., 2004).

Present research work was planned to evaluate the activity of antioxidant enzyme superoxide dismutase in kidney of silver carp under the exposure of heavy metal mixture (Pb+Cr).

MATERIALS AND METHODS

Experimental fish

Silver carps were purchased from Faisalabad hatchery to check the chronic effect of (Pb+Cr) on the antioxidant enzyme superoxide dismutase from kidney of silver carp. For this one group of fish kept under chronic exposure of metals mixture (Pb+Cr) for 15 days and other group of fish was present in unstressed conditions.

Temperature (30°C), hardness (230 mgL^{-1}) and pH (7.5) were kept constant throughout the experimental period.

Isolation of SOD enzyme

Desired organ (kidney) was separated after fish dissection. Phosphate buffer having pH 6.5 was used to homogenize the organ and centrifuge at 10,000 rpm for 15 min at 4°C. Supernatants were separated for further analysis.

Enzyme assay

The activity of superoxide dismutase was determined by measuring its ability to inhibit the photoreduction of Nitrobluetetrazole (NBT) following the method of Giannopolitis and Ries (1977). One ml buffer was taken in cuvette as blank and inserted into spectrophotometer to note the readings of blank, after taking reading spectrophotometer was adjusted at zero at A_{560} nm. Then 5-6 cuvettes were taken and set them in a light box with an internally mounted light bulb of 30 Watt. Firstly 1 ml of buffer was added to each cuvette, then 0.05 ml enzyme extract and 0.016 ml of riboflavin was added in each cuvette. All the cuvettes were incubated in light box for 12 min. The cuvettes were transferred to the spectrophotometer, where 0.067 ml of EDTA/NaCN solution and 0.033 ml of NBT was added to the illuminated reaction mixture. The absorbance was noted after 20 s of reaction.

Purification of SOD enzyme

Crude enzyme was partially purified by ammonium sulphate precipitation using the method of Shin *et al.* (1993). Ammonium sulfate precipitations further consist of Salting-in (60%) and Salting-out (80%) steps. The whole of the dissolved sample was dialyzed in dialysis bag, while continuous stirring in buffer with pH 7.8.

After dialysis of the test sample the enzyme was purified by ion exchange chromatography following the method of Zia *et al*. (2007). The column of DEAE-cellulose was used.

Characterization of SOD enzyme

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 (0, 20, 30, 40, 50, 60, 70 and 80°C) keeping the pH 6.0.

Statistical analysis

Data obtained was analyzed by using Minitab software. MS Excel was used to draw graphs.

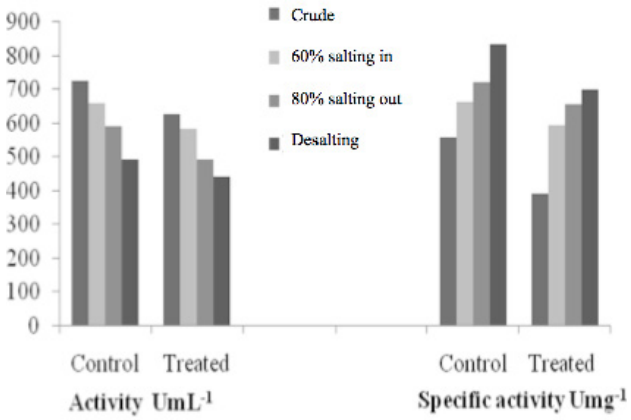

Fig. 1. Partial purification of SOD enzyme by ammonium sulfate precipitation.

RESULTS

Partially purified SOD enzyme

Activity of enzyme is the amount of micromole of a substrate to transform into product by enzyme per mL per minute under standard conditions. In present study, results of crude showed that the activity of SOD was decreased in kidney of Pb+Cr exposed fish (625 UmL^{-1}) as compared to control (725 UmL^{-1}) (Fig. 1). SOD was partially purified through ammonium sulphate precipitation. Specific activity of enzyme is defined as "units of enzyme per milligram of protein". In result of 80% saltingout the SOD activity was 591 and 491 UmL^{-1} while specific activity was 721 and 655 Umg^{-1} in controlled and Pb+Cr treated fish, respectively. In this study after desalting, activity of kidney SOD was estimated as 491.67 and 441.66 UmL^{-1} in controlled and Pb+Cr treated fish, respectively. Specific

activity was calculated as 833.33 and 701.04 Umg^{-1} for control and Pb+Cr treated fish. After the process of desalting the partially purified enzyme was used for ion-exchange column chromatography. Data regarding to ammonium sulphate precipitation are given below graphically.

For purification of SOD enzyme 50 fractions were taken from column for both control and treated sample. As a result of ion exchange chromatography enzyme from kidney of control and treated fish had highest activity as 508.33 and 427 UmL^{-1} while highest specific activity was 1105.06 and 1055.55 Umg^{-1} at 28th and 21st fraction, respectively (Table I). Fold purification is a "measure of times purification that an enzyme is how many fold purified" so enzyme from kidney of control and treated fish was 1.98 and 2.70 fold purified, respectively. Percentage recovery was 70 and 68%, respectively. Result showed in each and every step of purification, that the activity of SOD was decreased while specific activity increased from crude to ion-exchange chromatography. Results were statistically significant at $p<0.01$. Both 28th and 21st fraction were used for further characterization.

Table I.- Summary of ion-exchange chromatography for SOD.

Control			Treated (Pb+Cr)		
Fraction	Activity (UmL^{-1})	Specific activity (Umg^{-1})	Fraction	Activity (UmL^{-1})	Specific activity (Umg^{-1})
9	258.33	469.69	2	291.67	620.57
21	325.00	633.26	7	383.33	851.84
23	366.66	833.31	11	408.33	1047.00
28	508.33	1105.06	21	427.00	1055.55
31	483.33	1074.06	27	366.67	1047.62
33	466.66	1048.67	32	341.66	1007.84

Characterization of SOD enzyme

Effect of PH

Kinetic study has demonstrated that enzyme had a broad range of pH from 4 to 8.5. It was observed that enzyme from kidney of control and stressed *H. molitrix* had highest activity at pH 7.5 and 6.5 (517 and 570 UmL^{-1}, respectively) (Fig. 2).

Effect of temperature

It was observed that activity of enzyme increase with increase in temperature but up to a certain limit. It was also observed that treated sample had more tolerance than controlled. The optimum temperature at which maximum activity obtained (525 and 550 UmL^{-1}) was 40°C and 50°C for kidney of control and stressed fish, respectively (Fig. 3).

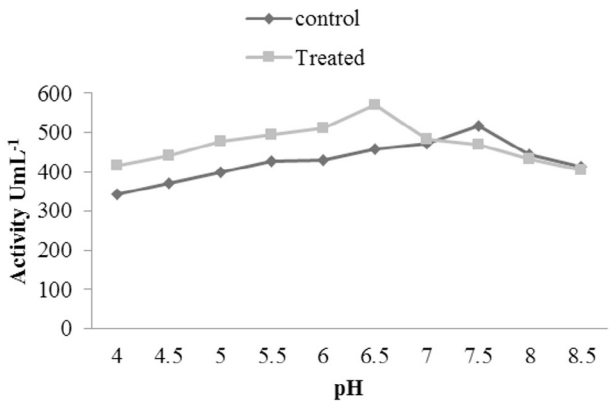

Fig. 2. Effect of different pH on activity of SOD.

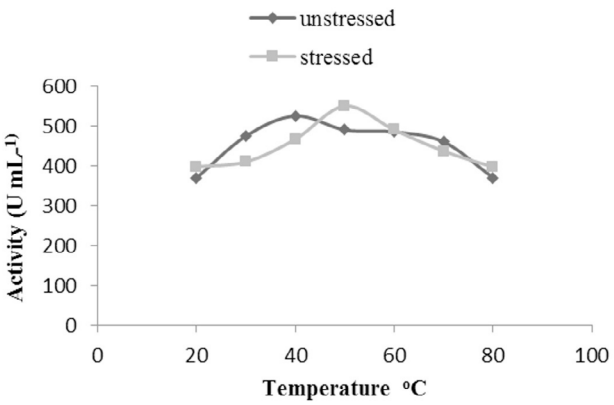

Fig. 3. Effect of different temperature on activity of SOD.

DISCUSSION

Metals are not only essential constituent of ecosystem but also used as food source and are capable of inducing biochemical and physiological changes in fish (Atli and Canli, 2008). Aquatic life is more susceptible to heavy metals toxicants include Cd, Pb, Cr and Cu and long term contact with these metals induce oxidative damage by producing free radicals (Doherty *et al*., 2010). Fish organs like liver and kidney are gifted with antioxidant defense mechanism to prevent them from oxidative stress caused by metals (Atli and Canli, 2008). Kidney is important organ because of its function in elimination of excess water, regulate acid-base balance, control ions and maintain urea (Holzer *et al*., 2004).

Superoxide dismutase is an antioxidant enzyme which plays a vital role in scavenging of free oxyradicals

(Li *et al*., 2010) by converting superoxide anion radical (O^{2-}) into H_2O_2 (Ozmen, 2005). Present research work was done to check the effect heavy metals mixture on SOD enzyme in kidney of silver carp. Results indicated that the activity of SOD was decreased in kidney of silver carps under chronic exposure of metal mixture (Pb+Cr) when compared with control fish. These results are also supported by Atli and Canli (2010) who demonstrated that antioxidant systems are most susceptible to metal exposure. They also suggested that there is a link between responses of enzymes and duration of metal exposure. These responses also associated with organ and exposure types. Sub-lethal concentrations of metals mixture generally inhibit superoxide dismutase (SOD) in kidney of *Oreochromis niloticus*.

Under the chronic exposure of lead, a significant decline in SOD activity was observed in the kidneys (23.4%) of *Crucian carp* exposed to 30 μgL^{-1} of lead (Khan *et al*., 2015). Our results are also supported by previous study concerning with chromium toxicity. Chromium (III) reduced the SOD activity 30% in kidney of goldfish (Lushchaka *et al*., 2009). Adeogun *et al*. (2012) reported that the activity of superoxide dismutase showed no significant increase in kidney of *Clarias gariepinus* under chronic exposures of binary mixtures of industrial effluents. Heavy metals induced oxidative stress by altering the antioxidant enzyme in aquatic organisms. Oxidative stress can be used as a biomarker to assess the aquatic contamination (Farombi *et al*., 2007). Velma and Tchounwou (2013) investigated the toxic role of chromium in oxidative stress (liver and kidney) of gold fish. They concluded that the heavy metal chromium induced toxic affect in liver and kidney but kidney is more sensitive to this toxicity. Previous study revealed that liver and kidney have high concentrations of heavy metal (Cd and Pb) led to decreased SOD activity. Increases in metal concentrations in the aquatic environment simultaneously reduced SOD activity (Brucka-Jastrzebska, 2010).

The values of ammonium sulphate precipitation salting in and salting out for SOD are 60% and 80%, respectively (Pedrajas *et al*., 1993). The Isoelectric point of the superoxide dismutase was 4.75 reported by Carrico and Duetsch (1970). According to Briggs and Fee (1978) molecular mass of SOD was 32.0 kDa. An optimum pH is required for the enzymes to work normally (Nelson and Cox, 2008). Keele *et al*. (1971) reported the optimum pH for superoxide dismutase as 7.8 and the pH range for SOD at which it remain active was 7.6-10.5 (Rigo *et al*., 1978). There was no work present on purification and characterization of SOD from fish but Aydemir and Tarhan (2001) was used column which contain DEAE-cellulose and Sephadex G-100 gel, for purification of SOD. Specific activity of SOD from erythrocytes of chicken was 8,480 Umg^{-1}. Results of characterization showed that enzyme work within range of pH 7.0-9.0 and at 25°C had maximum. The value of specific activity in the purification of SOD was 2000 Umg^{-1} (Ken *et al*., 2003).

CONCLUSION

The present research work concluded that heavy metal exposure can induce the production of ROS in silver carp under sub-lethal test concentration. And result in significant decrease in activity of antioxidant enzyme instead of improving yield. Results suggest that antioxidant defense systems can be used as sensitive biological indicator for detecting the effect of aquatic pollution in fish.

Statement of conflict of interest

Authors have declared no conflict of interest.

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