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Natural Occurrence of Metallothionein-Like Proteins in the Liver of Fish *Oreochromis niloticus* and Effects of Cadmium, Lead, Copper, Zinc, and Iron Exposures on Their Profiles

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Metals present in the aquatic environment have been seen as a potential treat for aquatic organism for several decades. Studies on heavy metal uptake and toxicity have shown that fish accumulate metals in their tissues and toxic effects may occur depending on exposure concentrations, durations and species in concern (Roesijadi and Robinson 1994; Heath 1995), especially the early life stages of fish development are more sensitive to metal contamination (Weis and Weis 1989). Natural waters are occasionally monitored to understand the current heavy metal load. However, it is important to find a sensitive measurement before toxic effects occur in fish metabolism.

Metallothionein (MT) could be a sensitive indicator of heavy metal contamination in the aquatic environment and this has been suggested by several authors, especially for cadmium contamination (Roch et al. 1982; Haux and Förlin 1988; Hogstrand and Haux 1990; Dallinger et al. 1997; Canli et al. 1997; De Conto Cinier et al. 1998). MTs are ubiquitous low molecular weight (6000-7000) proteins that are rich in cystein contents, heat-resistant, absent in aromatic amino acids and disulfid bonds and selective capacity to bind heavy metals such as cadmium, copper, zinc and mercury (Kägi and Schäffer 1988; Roesijadi 1992; Roesijadi and Robinson 1994). MTs occur naturally in tissues and serve as potential donor for the essential metals and also they bind non-essential metals for sequestration and both metal groups situmulate MT synthesis, though levels of induction may differ (Roch et al. 1982; Brown et al. 1987; Hogstrand and Haux 1990; Roesijadi 1992; Roesijadi and Robinson 1994; Canli et al. 1997; Wu et al. 1999).

Tilapia (*Oreochromis niloticus*) are widely distributed freshwater fish that can persist in a highly polluted habitat and have the potential for the development as a biological monitor of environmental pollution (Ueng et al. 1996). The aim of this study is to describe natural occurrence of metallothionein and other metal binding proteins in the liver of *Oreochromis niloticus* and investigate their induction in relation to essential (Cu, Zn, Fe) and non-essential (Cd, Pb) metal exposures.

MATERIALS AND METHODS

Freshwater fish *Oreochromis niloticus* were supplied from fish culturing pools at Çukurova University. They were transferred to the laboratory where the experiments were carried out. Before the experiments, fish were acclimatized to the laboratory conditions for one month. The experimental room was air conditioned (20±1.1 ^{o}C) and illuminated with two fluorescent lamps (daylight 65/80 W) for 12 hours. The tap water

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used for the experiment had a pH value of 8.32±0.8 and a total hardness of 340±4.54 mg $CaCO_3$/L. The aquariums were aerated with air stones attached to an air compressor to saturate with oxygen (6.01±0.44 mg O_2/L). The experiments were conducted in glass aquariums sized 33*33*40 cm, each containing 4 fish in 30 L of contaminated test solution or tap water only (for controls). Fish were exposed to 1 mg/L concentration of lead ($Pb(NO_3)_2$), copper ($CuSO_4.5H_2O$), cadmium ($CdCl_2.H_2O$), zinc ($ZnCl_2$) and iron ($FeCl_2.4H_2O$) for 10 days. Waters in the control and metal containing aquariums were changed every two days to minimize metal loss. Water was changed just after feeding the animals, thus preventing contamination of the environment with food remains.

At the end of the 10 days exposure period, all fish from three replicate tanks were taken out and killed by a blow to the head and their total lengths were measured to the nearest mm. Mean lengths of fish (109 ± 15.4 mm) did not differ significantly ($P>0.05$) among different exposure regimes and control. After the exposure to metals for 10 days, animals were dissected with clean equipments to obtain liver tissues. Liver samples from 12 fish of the same exposure group were pooled and mixed well. Mixed livers were allocated for the determination of total metals, proteins and gel filtration. All tissues were frozen at –20 °C for a few weeks before the analyses.

The same amount (1 g) of liver tissue for each experiment and control were homogenized in 10 ml buffer containing 40 mM Tris-HCl, 0.25 M sucrose and 5 mM EDTA (pH 7.4). Homogenates were centrifuged at 2500 g for 10 min. The supernatants were heated in water bath at 80 °C for 10 min. Coagulated particles were removed by centrifugation at 2500 g for 10 min and supernatants were collected for gel filtration. A column (2.7 x 60 cm) packed with Sephadex G-75 and an elution buffer containing 50 mM Tris (pH 8.0) and 3.1 mM sodium azide was used for gel filtration (elution rate of 1.67 mL/min). 5 mL eluate was collected in test tube for the measurements of metals, sulfhydryl groups and protein absorbance. A total of 100 tubes was obtained for each gel filtration process. A series of molecular weight markers was used to measure approximate molecular weight of proteins in eluates. Albumin (66000), carbonic anhydrase (29000), cytochrom c (12400) and aprotinin (6500) were used as molecular weight markers. Linear Regression analyses carried out using elution volume and molecular weight of the markers and following equation ($y = 5.77-0.65x$, $r^2 = -0.96$) was found.

Sulfhydryl group determination was carried out using the Ellman method (Robyt and White 1987). Ellman's reagent (5,5'dithiobis [2-nitrobenzoic acid]) was used as a standard method for the quantitative determination of reactive sulfhydryls in proteins. Measurement of total protein in the crude homogenates was carried out using the Lowry method (Lowry et al. 1957). Proteins in eluates were determined directly measuring absorbance at 254 and 280 nm. Total metals in the liver and metals in eluates were measured with the method explained elsewhere (Canli 1995) using an atomic absorption spectrophotometer (Perkin Elmer AS 3100) that works with air-acetylene flame. The detection limits of the metals were 0.002, 0.004, 0.02, 0.001 and 0.002 (µg/mL) for Cu, Fe, Pb, Cd and Zn respectively.

RESULTS AND DISCUSSION

In this study, 1 µg/mL concentration of Cd, Pb, Zn, and Fe was not lethal for *Oreochromis niloticus* in 10 days though the same concentration of Cu showed lethal

effect after 6th day. Copper toxicity in fish is well known and previous studies also showed its toxic effect in low levels (Collvin 1985; Lauren and McDonald 1987; Ay et al. 1999). Generally, copper is among the most toxic heavy metals together with mercury, arsenic and silver and these were followed by cadmium, lead and zinc (Bryan 1971; Heath 1995). It is well known that metal accumulation occur in fish liver in relation to exposure concentration and duration, though some parameters of water such as temperature, salinity, hardness and pH can also affect accumulation levels of metals (Heath, 1995). In this study, mean concentrations (µg metal/g. d.w.) of zinc, copper, iron, cadmium and lead in the liver of *O. niloticus* were found as 81.88 (96.15), 349.4 (466.2), 84.06 (102.7), 4.26 (32.66) and 43.67 (248.2) in control and exposure experiments (in parenthesis). All metals accumulated in the liver, though levels of accumulation differed greatly among metals. It seems that only zinc levels were similar between control and the exposure experiment, suggesting its regulation by the fish. Total protein levels in the control liver (24.5 µg/g w.w.) did not differ more than 10 % when compared to protein levels in the liver of fish exposed to metals.

The elution profiles of both heat-treated (80 °C) and normal controls were measured at 254 and 280 nm for proteins and 412 nm for sulfhydryl groups and were presented in Figure 1 and 2. These figures show that liver cytosol of *O. niloticus* produced three protein peaks. The first peak obtained between 14-21 fractions and contained high molecular weight proteins (HMWP) greater than 70000 Da. These proteins contained sulfhydryl groups and also bound zinc (4.75 µg/mL), copper (0.96 µg/mL) and iron (0.28 µg/mL). However, these proteins and metals bound on them were largely removed in the heat-treated control (Figure 3). This clearly indicates that they are not MT-like proteins. The second peak obtained between 22-37 fractions and contained medium molecular weight proteins (MMWP) approximately 43000 Da. This peak remained in heat-treated samples and bound both copper and zinc. However, these proteins did not contain sulfhydryl groups that mean they are also not MT-like proteins. The third peak obtained between 38-60 fractions and contained low molecular weight proteins (LMWP) approximately 6500 Da. Heat-treatment did not alter their profile. Proteins in this peak bound both copper and zinc and contained sulfhydryl groups. Thus, they are called MT or MT-like proteins. Heat-treatment of tissue samples is an important process in MT studies because of interactions of other metal-binding proteins that also contain sulfhydryl groups. Thus, in many studies heat-treatment was used to determine accurately MTs or other metal-binding proteins in tissues of aquatic animals (Shears and Fletcher 1985; Viarengo et al. 1985; Canli 1995; De Conto Cinier et al. 1998; Wu et al. 1999).

Metallothioneins are low molecular weight (6000-7000), heat-resistant proteins that are characterized by unusually high cystein content, lack of aromatic amino acids and selective capacity to bind heavy metals such as mercury, cadmium, copper and zinc. MTs are naturally present in animal tissues as storage form of the essential metal (e.g. Cu and Zn) and play conspicuous roles in the extracellular and intracellular control of copper and zinc metabolism. Thus, the basal levels of MTs are considered to be involved in the essential metal regulation. MTs can also be induced by several factors, most important inducers being group 1B and 2B metals (Kägi and Schäffer 1988; Viarengo 1989; Roesijadi 1992). There is no study to our knowledge on lead and/or iron binding MT-like proteins in the literature (Reichert et al. 1979; Roesijadi 1992; Roesijadi and Robinson 1994). Similarly, the present study also did not reveal such proteins. In this study, heat-resistant (80 °C), low molecular weight (6500) proteins that

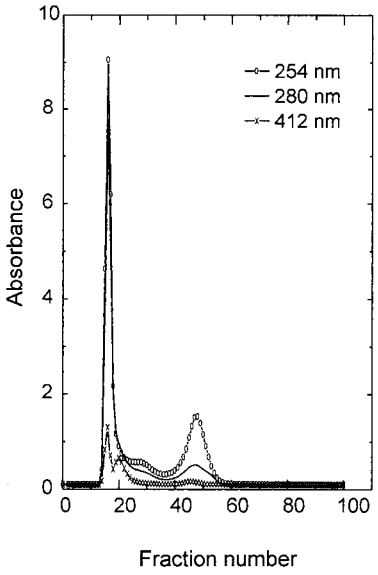

Figure 1. Sephadex G-75 elution profile of liver homogenate from control *O.niloticus*.

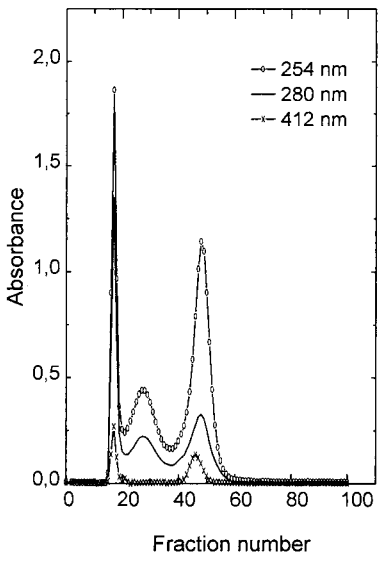

Figure 2. Sephadex G-75 elution profile of heat-treated (80 ^{0}C) liver homogenate from control *O. niloticus*.

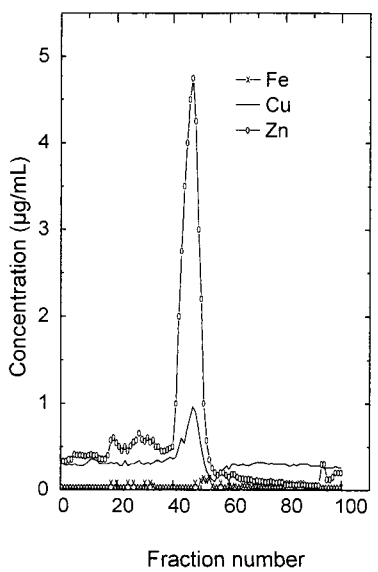

Figure 3. Metal distrubition in eluats of heat-treated (80 ^{0}C) liver homogenate from control *O. niloticus*. Cd and Pb levels were below the detection limits

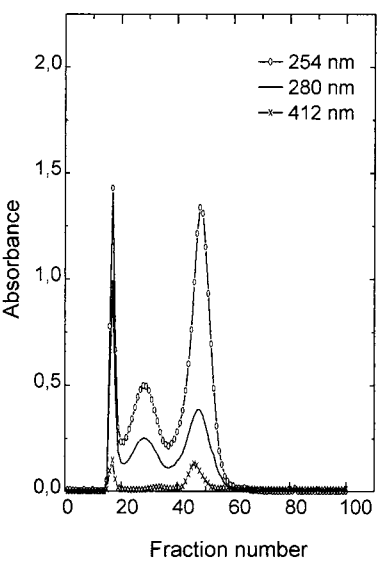

Figure 4. Sephadex G-75 elution profile of heat-treated (80 ^{0}C) liver homogenate from Cd-exposed *O. niloticus*.

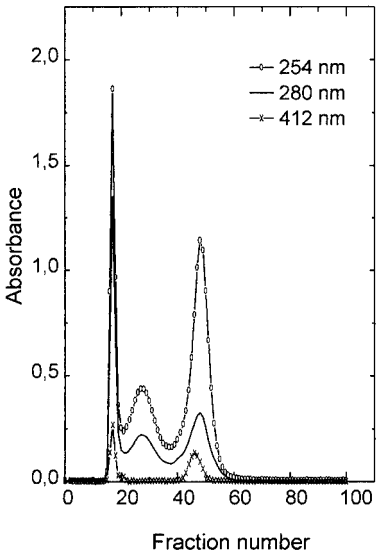

Figure 5. Sephadex G-75 elution profile of heat-treated (80 ^{0}C) liver homogenate from Zn-exposed *O. niloticus*.

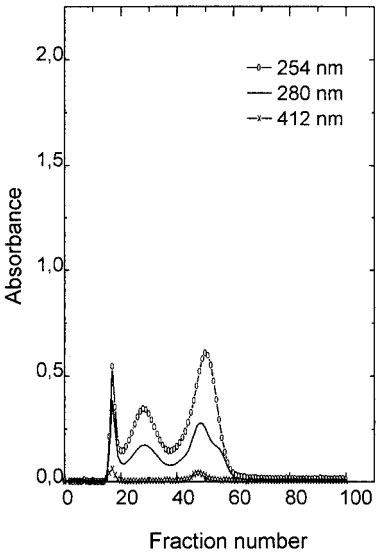

Figure 6. Sephadex G-75 elution profile of heat-treated (80 ^{0}C) liver homogenate from Cu-exposed *O. niloticus*.

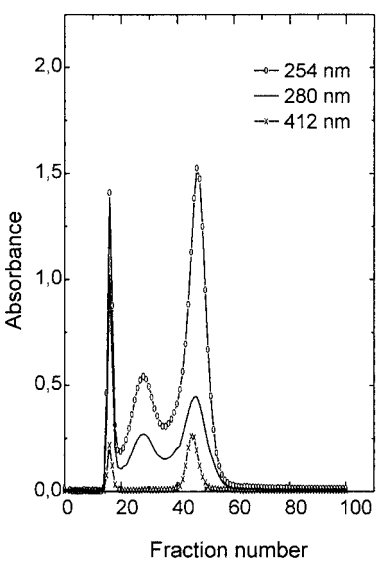

Figure 7. Sephadex G-75 elution profile of heat-treated (80 ^{0}C) liver homogenate from Pb-exposed *O. niloticus*.

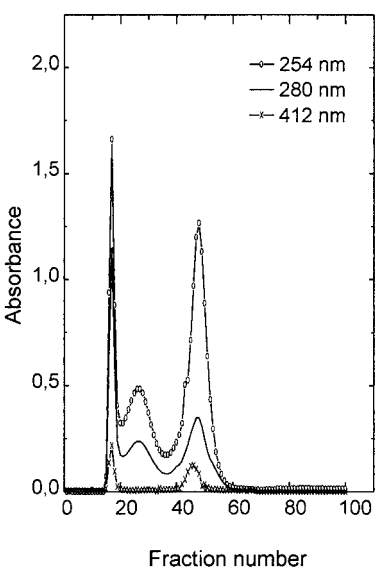

Figure 8. Sephadex G-75 elution profile of heat-treated (80 ^{0}C) liver homogenate from Fe-exposed *O. niloticus*.

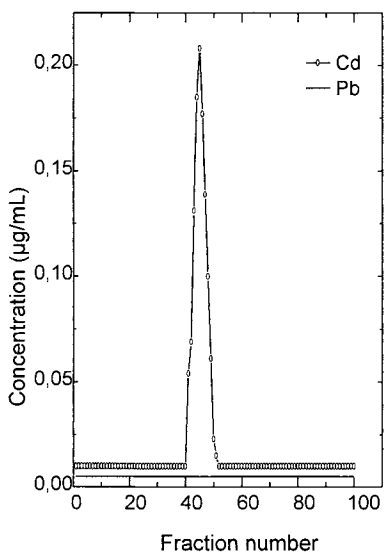

Figure 9a. Cd and Pb distribution in eluats of heat-treated (80 ^{0}C) liver homogenate from Cd and Pb exposed *O. niloticus*. Pb level was below the detection limit.

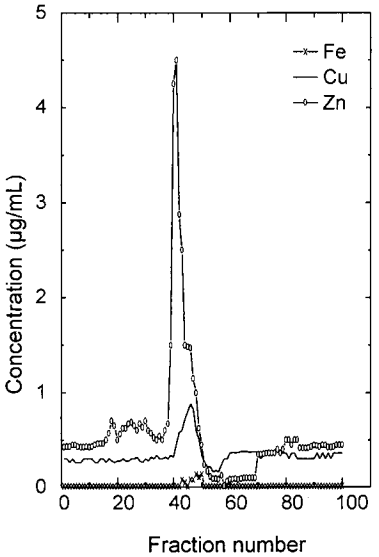

Figure 9b. Cu, Zn and Fe distribution in eluats of heat-treated (80 ^{0}C) liver homogenate from Cu, Zn and Fe exposed *O. niloticus*.

bind metals (Cu, Zn, Cd) and contain sulfhydryl groups were demonstrated in the liver of *O. niloticus*. Natural occurrence of copper and zinc MTs was shown in tissues of fish, though cadmium MTs were absent naturally in control animals or animals from the field (Olsson and Hogstrand 1987; Brown et al. 1987; Canli 1995; Ueng et al. 1996; De Conto Cinier et al. 1998; Wu et al. 1999) unless they were caught from a contaminated site (Hogstrand and Haux 1990; Dallinger et al. 1997; Olsvik et al. 2001).

The elution profiles of heat-treated and metal exposed fish were shown in Figure 4-9. Like controls, the liver of metal exposed fish also produced three peaks at 254 and 280 nm, and two peaks at 412 nm of which correspond to HMWP and LMWP. In metal exposed groups, lead and iron did not correspond with any of the peaks obtained whereas copper and zinc bound on these proteins. Cadmium bound only on LMWP when fish exposed to this metal (Figure 9a and b). The elution profile of liver from *Oreochromis mossambicus* was found to be similar to the profiles found in the present study (Wu et al. 1999). They found that all three proteins peaks were contained thiol groups and high molecular weight fraction also corresponded with cadmium after exposure to this metal. In the present study, however, cadmium did not bind on HMWP. Olsson and Hogstrand (1987) studied subcellular distribution of cadmium in tissues of rainbow trout *Salmo gairdneri*. They found three protein peaks in the liver and among these peaks only MT fraction contained cadmium. Unlike cadmium, copper and zinc on MTs do not increase sharply in low environmental exposure concentrations. This may be due to high background levels of these metals in tissues. Olsson and Haux (1986) showed that copper and zinc levels did not increase significantly with increases in

exposure levels in the liver of perch *Perca fluviatilis*. McCarter et al. (1982) also observed that levels of copper in LMWP fraction of the liver in coho salmon did not increase significantly after 6 weeks of exposure to this metal. Similarly copper and zinc concentrations in MT fraction of hepatopancreas from *Nephrops norvegicus* did not increase significantly after exposure to these metals (Canli 1995). Copper and zinc bound on MT or MT-like proteins in aquatic animals can also increase in environments that are excessively contaminated or in animals that lived for a long period in metal contaminated mediums (Hogstrand and Haux 1990; Roch et al. 1982). Nevertheless, studies from the literature generally showed that MT concentrations increased, regardless of their metal content, in tissues of fishes when they are exposed to metals given via water, food or injection (Bonham and Gedamu 1984; Hogstrand and Haux 1989; Ueng et al. 1996; De Conto Cinier et al. 1998; Wu et al. 1999; Dang et al. 2001).

The figures presented in this study show that the proportion of HMWP peak to LMWP peak differed considerably between control and metal exposed fish. For example, the peak of HMWP in control was approximately 25 % higher than the peak of LMWP (Fig 3), while in metal exposed groups those two peaks were closer to equal (Fig 4-8). The profile of copper exposed group was somewhat distinguishably different than the others, as the HMWP and LMWP peaks were very low when compared the peaks from the other metals. However, copper levels, interestingly, did not differ considerably between control and copper exposed group. As mentioned earlier, copper was the only metal that caused mortality after 6 days of exposure to 1 µg Cu/mL. It is well known that one of the roles of MT-like proteins is to protect organism against the toxic effects of metals when they are excessively present in the medium. Binding on these proteins would prevent metals to become intercepted with some vital enzymes (Roesijadi and Robinson 1994; Heath, 1995). Copper toxicity and the profile of copper exposed fish in this study suggest, on the one hand, that the induction of MT-like proteins in the liver of copper exposed fish was very low due to high basal level. On the other hand, it also suggests that the ratios of metals and proteins in MT pool might be affected adversely by copper exposure causing toxicity and low protein absorbance. The behavior of metals in MT pool might be enlightened in detail by using better protein purification techniques and more sensitive measurements of metals in the liver.

In conclusion, this study revealed the natural occurrence of copper and zinc MTs but not cadmium, lead and iron MTs in the liver of *O. niloticus*. Exposure of fish to these metals in the same concentration showed that only cadmium associated with MT fraction. The levels of copper and zinc in MT fraction did not increase considerably, though this may not mean that Cu and Zn did not bind on MTs after exposure to these metals. Cadmium accumulation could be measured in MT pool because the control level was below the detection limit. However, basal levels of Cu and Zn in MT pool in control were already high and a similar increase of these metals in MT pools could not be measured sensitively in metal exposed groups. Lead was never associated with any of the proteins in both control and metal exposed groups. Similarly, iron did not also bind on proteins considerably especially after the heat-treatment. Thus, this study indicates that measurement of metals in MT pool can produce sensitive data only for cadmium and Cd-MTs can be a good indicator of environmental cadmium contamination in the liver of *Oreochromis niloticus*.

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