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METALS EVALUATION IN CYTOSOLS AND LIVER EXTRACTS AND LIKE-METALLOTHIONEIN (MT) PURIFICATION IN THE CATFISH *Cathorops spixii*

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ABSTRACT

Several metalloproteins have been proposed as biological indicators of pollutant exposure. Of these, the metallothioneins are great as exposure biomarkers to environmental contamination by metals. Pb, Cd, Cu and Mn were evaluated in this work since are release in the Santos-São Vicente estuary for consequence of the different industrial activities. Previous work demonstrated that the Ariidae catfish *Cathorops spixii* is efficient bioindicator for metal contamination. In this context, metals contents in liver and cytosols were determined by HR-ICP-MS. In order to obtain the like-metallothionein (MT), hepatic cytosols were subjected to size-exclusion (SE-HPLC) with protein elution followed by on-line UV/Vis detection. Conditions of analysis were performed as mobile phase of 10 mM Tris pH 7.4, flow of 0.5 ml mim^{-1} and λ of 254 nm. The results showed that the protocol establish was possible the purification of the like-MT of *C. spixii*. Zn, Cu and Mn were the most abundant metals in the total liver (Zn: 42,369 µg Kg^{-1}; Cu: 6,882 µg Kg^{-1}; Mn: 897 µg Kg^{-1}; Cd: 161µg Kg^{-1}) than cytosols fractions (Zn: 5,970 µg Kg^{-1}; Cu: 32 µg Kg^{-1}; Mn: 11 µg Kg^{-1}; Cd: 1 µg Kg^{-1}). In fact, metals and like-MT data are important indicative of the MT detoxification process in *C. spixii* from polluted areas.

1. INTRODUCTION

Even at the level of individual enzymes, most foreign chemicals inhibit or stimulate several enzymes, although some enzymes are more sensitive than other to a given chemical [1]. Metallothionein (MT) is a low-molecular-weight protein which has many sulfhydryl groups due to the large amount of cysteine in the molecule. These sulfhydryl groups bind a variety of metals and therefore, presumably, make them less toxic to other cellular constituents. There may be several organs where MT is functional in fish, but the liver has thus far received the bulk of attention. With the tendency for the liver to accumulate several metals, the association of large amounts of MT with this tissue is not surprising. [1]

Santos/São Vicente estuary is an important economic area in Brazil. Besides tourism, the largest commercial harbor of South America operates in the city. Additionally, the most important petrochemical and metallurgical complex, composed of more than 1100 industries, is located in the region. Cananéia is an important estuarine system located in the North of the São Paulo State, Brazil, due has extensive environmental protection areas. Furthermore, this aquatic system is very used as reference site in biomonitoring studies due their low human influence.

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Ariidae *Cathorops spixii* have a wide geographical distribution along the Atlantic coast of South America, ranging from Belize to the Southern Brazilian coast. *C. spixii* is the most common catfish in the Brazilian Coast [2]. This benthic fish is eurihaline species, feeding mainly of materials and organisms within the mud (silt and clay), where the bioavailability of contaminants is higher [3]. However this species is used to monitor the effects of toxic metal pollution in estuaries of the Brazilian coast [4, 5, 6], at the moment, there is an absence of information about the MT characterization. In this study, like-metallothionein (MT) purification was carried out in the catfish *C. spixii*.

In order to understand some aspects of the detoxification process in *Cathorops spixii*, Zn, Cu, Cd and Mn were determined in the total liver and cytosol extracts. Identification and purification of the like-MT were also subjected of this work.

2. MATERIAL AND METHODS

2.1. Instrumentation

A high resolution inductively coupled plasma mass spectrometer HR-ICP-MS (Element, *Finnigan*) was used for metals measurements. Instrumental operating conditions for the HR ICP-MS are summarized in Table 1.

An Ultra-turrax system (Potter, CT-136) was used for tissue homogenization. Liver cytosol was obtained by ultracentrifugation using a Beckman ultracentrifuge (Beckman Coulter, USA). A Superdex peptide HR 10/300 column (GE Healthcare, Sweden) was used to perform size-exclusion (SE)-HPLC. Shimadzu LC-10Ai HPLC pumps were used as the solvents delivery system and injections were done using a sample injector valve with a 100 µL loop. A UV-Vis detector model SPD-10AVi variable wavelength detector was used for spectrophotometric measurements.

Table 1.Operating conditions for HR-ICP-MS instrument.

Carrier gas flow rate	0,9 l mim^{-1}
Rf power	1250 W
Internal standard	In^{115} 5 and 1 µg Kg^{-1}
Run and pass	4 and 5 times
Isotope (300 resolution)	Cd^{111}
Isotopes (3700 resolution)	Mn^{55}, Cu^{63}, Zn^{66}

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2.2. Reagents and materials

All reagents used were of analytical grade purchased from Merck. Water (18 mΩ) prepared with Mili-Q system was used throughout. Multiement stock solutions were prepared from sub-boiled (65%) nitric acid and Milli-Q water. All dilution were performed by mass and prepared daily. MT-1 standards from rabbit liver were purchased from Alexis Biochemicals (USA).

2.3. Fish sampling

C. spixii were collected in two consecutive sample periods during July and August 2009 using gill nets of 20 mm mesh. Individuals were collected from two estuaries: one polluted (Santos-São Vicente) and either with low human influence (Cananéia). After collection, the fish were identified in accordance with Figueiredo and Menezes [2], anesthetized with benzocaine (2% in water), killed by spinal section and transported to the laboratory. Liver samples from each specimen were removed, packed in polyethylene bags, and stored at -80°C until analysis.

2.4. Cytosol preparation and metals analysis

Hepatic cytosolic extracts were obtained for ultracentrifugation. Briefly, livers were homogenized in 10 mM Tris-HCl, pH 7.4 and centrifuged three times (1000 g to 10 min; 105,000 g to 60 min; and 105,000 g to 30 min). Cytosols were diluted (1:5) with sub-boiled 2% nitric acid and contents of Zn, Cu, Cd and Mn measured using a HR-ICP-MS. 1 μg Kg^{-1} Indium was used with internal standard. For total hepatic metals determinations, 0.2 g of sample were placed in pre-weighed 100 mL Teflon tubes. 65% HNO_3 and 30% H_2O_2 were added and digested by heating in a microwave (CEM Corporation, Mars 5 model) with parameters described below: 600 W, 100 %, 9 min of temperature ramp, 145 PSI, 145°C temperature and 5 min hold. Obtained solutions were diluted in milli-Q water until 25 mL and, diluted again (1:3) for HR-ICP-MS analysis. 5 μg Kg^{-1} Indium was used as internal standard. Blank samples and certified standard reference (Dogfish liver - DOLT-2 - National Research Canada Council, NRCC) were also analyzed in order to provide the analytical control and the obtained results are showed in Table 2.

Table 2. Metals concentration in standard reference material "Dog fish liver" (DOLT-2, NRCC) (n = 3) determined by HR ICP MS

Metal	Certified (mg Kg^{-1})	Obtained (mg Kg^{-1})	Recovery (%)
Cu	25.80 ± 1.10	20.45 ± 0.71	79
Zn	85.80 ± 2.50	82.90 ± 2.56	97
Mn	6.88 ± 0.56	5.80 ± 0.08	84
Cd	20.80 ± 0.50	18.69 ± 0.05	90

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2.5. Optimization of the hepatic MT isolation by size exclusion HPLC

Optimization of SE-HPLC parameters was carried out using standard solutions of MT liver rabbit. Tris-HCl in different concentrations was tested as mobile phase. Flow and injection volumes were also tested. The elution process of the MT from the SE-HPLC column was followed on-line by monitoring the absorbance at 254 and 220 nm by UV/Vis spectrophotometry.

3. RESULTS AND DISCUSSION

SEC is a very gentle separation technique where proteins and protein/metal complexes predominantly are separated according to their hydrodynamic radius (proportional to the molecular mass) under conservation of their native structure. The molecular mass of unknown substances can be estimated by means of column calibration with known molecular markers [7]. With a first step of optimization, were test consecutives volumes of injection: 10 µL, 25 µL, 50 µL, 75 µL, 100 µL, 200 µL and 500 µL of the hepatic cytosol samples. Volumes of 10 and 25 are not satisfactory to a good evidence of chromatographic profile. On the other hand, volumes higher than 75 µL not showed significant differences in the profile when compared to 50 µL. Therefore, were established a volume injection of 50 µL for the hepatic cytosol analysis by SEC.

Typical chromatographic profiles of consecutives injections of standard of purified liver metallothionein, using UV absorbance at 254 nm, are shown in Fig. 1. The magnification of the peak region shown that the elution time of MT is in 23 minutes. It is possible to observer a dose-dependent profile with good sensibility and reproducibly.

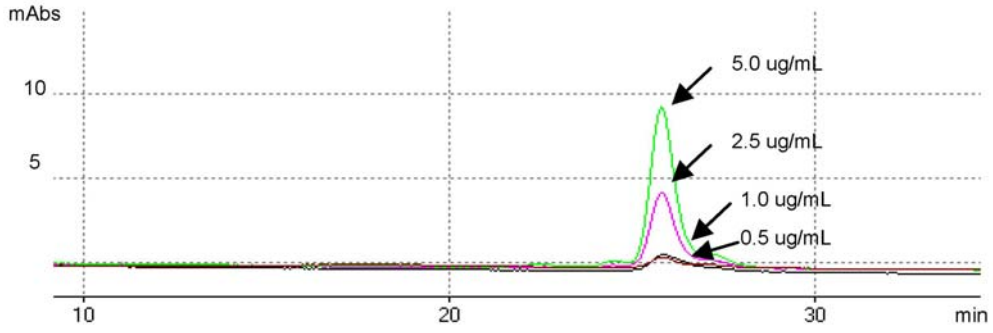

Figure 1. SE-HPLC profile obtained by UV/Vis spectrophotometric detection after increasing injections of the MT-1 standard (liver rabbit 6-7 kDa MW).

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Injections were monitored at 220 and 254 nm, since the first λ is the typical to absorption of peptide bonds and the absorbance 254 nm is characteristic to metal-thiolate bond absorption. Data about MT standard and hepatic cytosol of *C. spixii* profiles monitored in the both λ are showed in Fig. 2. A difference of circa of 40% was observed between profiles obtained to 220nm and 254 nm. The same profile was found when hepatic cytosol was analyzed. These results can be an indicative of the MT presence in the fraction obtained in this retention time because is known that MT is a protein rich in cysteine residues with an order between 40-60%.

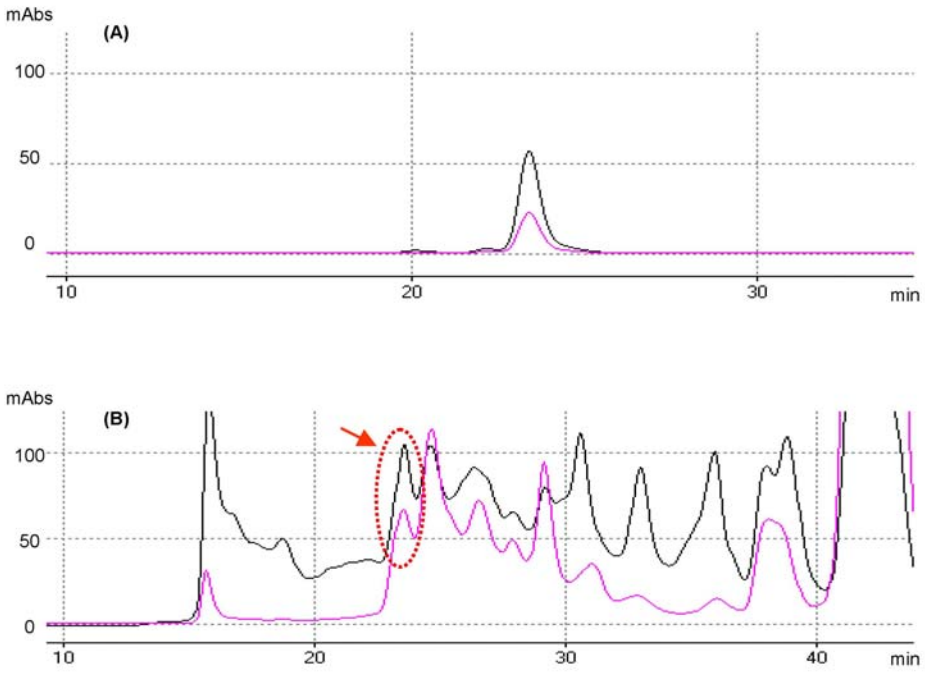

Figure 2. SE-HPLC profiles of MT standard (liver rabbit 6-7 kDa MW) (A) and hepatic cytosol sample of *C. spixii* (B) obtained by UV/Vis spectrophotometric detection to 200 nm (black line) and 254 nm (pink line).

Concerning the mobile phase, some concentrations of buffer were tested in order to optimize the conditions of separation by SEC. A pH 7.4 was established since it is the optimal pH of the activity of the metallothionein. Data regarding profiles of MT standard using 10 mM, 20 mM Tris-HCl and with addition with 100 mM NaCl were showed in the figure 3. Differences between 10 mM and 20 mM tris-HCl were not observed. On the other hand, when 100 mM NaCl was added in the Tris-HCl buffer it was observed a increase in the retention time of 23 min to 27 min. Therefore, the choice to mobile phase was to 10 mM Tris-HCl pH 7.4.

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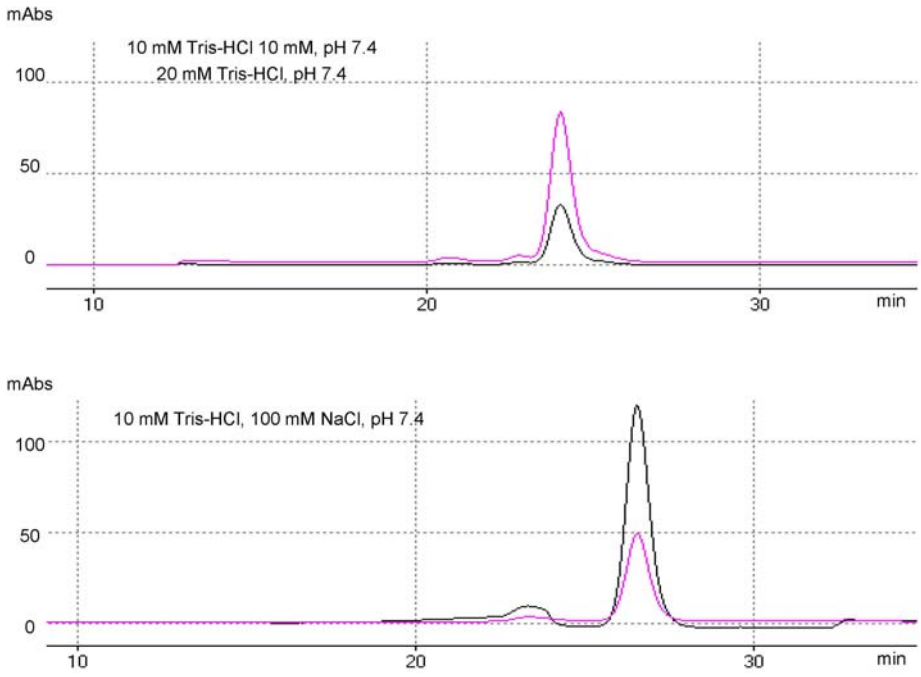

Figure 3. SE-HPLC profile by UV/Vis spectrophotometric detection of 50 ug mL^{-1} MT-1 standard (liver rabbit 6-7 kDa MW) with some mobile phases.

With results of the better volume injection, flow and mobile phase, was established the optimal conditions to analysis of the like-MT by SEC and these analytical data are showed in table 3.

Table 3. Optimization of chromatographic conditions by Size-Exclusion

Mobile phase	10 mM Tris-HCl, pH 7.4
Flow	0.5 mL mim^{-1}
Injection volume	50 uL
Time	50 min
λ	254 nm

Hepatic cytosols of *C. spixii* were applied in the size exclusion column and metals monitored off-line using HR-ICP-MS. In order to identify the elution time of the like-MT in the hepatic cytosols of catfish, were added a MT-1 standard in the cytosols samples and compared with

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samples without MT standard (Fig. 4). As can be observed, the absorbance profiles at 254 nm showed a peak occurring at a retention time of 23 min, which matches the retention time of

commercial rabbit liver MT standard. It is also possible to observer a increasing in this peak when MT standard was added in the hepatic cytosol samples of *C. spixii*. In association, there results are a strong indicative of the presence of like-MT in this retention time. It is important to consider the presence of others proteins with low molecular weight, since; in general, the SEC is an analytical method with low precision. Furthermore, the SEC is not capable of to discriminate particles with very close molecular weight. However, to purposes of purification and with a characteristic of partial identification, the SEC is very powerful and, therefore, was enough to achieve the goal of like-MT purification.

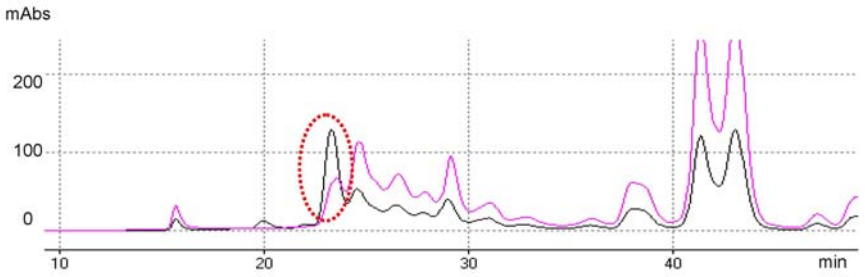

Figure 4. SE-HPLC profiles of hepatic cytosol samples of *C. spixii*. Pink line: samples without MT standard (liver rabbit 6-7 kDa MW); and Black line: sample with MT standard (liver rabbit 6-7 kDa MW) addition.

Data about metal analysis are show in the Table 4 and showed that Zn, Cu and Mn were the most abundant elements in the total liver (Zn: 42,369 µg Kg^{-1}; Cu: 6,882 µg Kg^{-1}; Mn: 897 µg Kg^{-1}; Cd: 161 µg Kg^{-1}). Similar profile was obtained to metals concentrations in the whole hepatic cytosol fractions (Zn: 5,970 µg Kg^{-1}; Cu: 32 µg Kg^{-1}; Mn: 11 µg Kg^{-1}; Cd: 1 µg Kg^{-1}). In fact, it is possible to observer metals levels between the total liver and the hepatic cytosolic fractions an availability of 14%, 0.5%, 1% and 0.6 to Zn, Cu, Mn and Cd concentrations, respectively in the cytosols, where are found proteins with low molecular weight such as like-MT.

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Table 4. Metals concentration (µg Kg^{-1}) and proteins of 6-7 kDa (like MT) from retention time of ~ 23 min in the cytosolic fraction in catfish collected in the both estuaries

	Cananéia	Santos-São Vicente
Retention time	23.69	24
Protein of 6 kDa (µg μL^{-1})	0.30	0.16
Zn	5175.52	5969.76
Cu	123.79	32.20
Cd	6.91	1.05
Mn	19.43	10.65

4. CONCLUSIONS

The results showed the presence of like-MT in comparison to standard MTs. Therefore, the protocol establish was enough to the purification of the like-MT in *C. spixii*. In fact, metals and like-MT data are important indicative of the MT detoxification process in *C. spixii* from polluted areas.

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