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Original Article

ISOLATION, PURIFICATION AND CONFIRMATION OF METALLOTHIONEIN FROM THE LIVER TISSUE OF CADMIUM EXPOSED FRESHWATER FISH, *CTENOPHARYNGODON IDELLA*

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ABSTRACT

Objective: Metallothioneins (MTs) are a group of low molecular mass, cysteine-rich proteins with a variety of functions including involvement in metal homeostasis, free radical scavenging, protection against heavy metal toxicity, and metabolic regulation. MT was purified from the liver tissue of cadmium (Cd) exposed fish (Ctenopharyngodon idella) using Affinity chromatography. While various chromatographic separation methods are available, although there exists various methods of separating low molecular weight proteins. Affinity Chromatography has been reported to be more suitable for separation as been confirmed by this study.

Methods: In the present study, in order to induce optimum MT concentrations in tissues, the experimental fishes were exposed to 5.0 ppm (which is about half the LC50 for this fish) of CdCl2 for 72 h. The induced MT protein was isolated and purified using Affinity chromatography (1 mL Hi-Trap metal chelating column) and desalting column (Sephadex G-25). Purified MT was evaluated by 15% SDS–PAGE and confirmed by Western blot with specific antibodies.

Results: MT was purified by Ni2+ loaded resins and eluted with 500 mM Imidazol. The elution was performed with 1 mL/min and the fractions were collected. Seven eluted fractions were obtained. The highest MT concentration was obtained in fourth fraction. Approximate molecular mass of MT was showed 6-7 kDa. Purified MT was confirmed by Western blot. The antibody shows a positive cross reactivity with MT protein.

Conclusion: MT can be used in bio-monitoring programs as a biomarker of Cd exposure in aquatic environments.

Keywords: Grass carp, Metallothionein, Cadmium, Affinity chromatography, Western blot.

INTRODUCTION

MTs were discovered by Margoshes and Valee [1] from a horse renal cortex tissue in 1957 [1]. These proteins are low-molecular mass (6-14 kDa), cysteine-rich proteins (18-/20 cysteines per molecule) lacking aromatic amino acids [2]. They are distinguished by an exceptionally high content of d10 metal ions (Zn^{2-}, $Cd^{2-}$and Cu^{-}) forming characteristic metal thiolate clusters through the sulphur atoms of all the cysteine groups that compose the protein, with thiolate groups acting both as a terminal and bridging metal ions [3]. MTs occurs in several isoforms which may differ from each other by only a few amino acid positions and by different isoelectric points and hydrophobicity [3, 4]. The existence of different MT isoforms with different roles has also been a matter of scientific interest [5].

The primary biological function of MTs is the storage, transportation, and replacement of trace elements [6] as well as the detoxification of heavy metals [7]. In addition, they have an obvious relationship in the regulation of hormones and of cell metabolism [8]. The function of this protein and its isoforms is still a matter under discussion. MTs have traditionally thought to be involved in the regulation of essential trace metals, such as copper and zinc and in the detoxification of essential and nonessential metals. More central functions are attributed to this proteins such as the intracellular scavenging of free radicals in protecting cells against oxidative stress [9] and zinc-mediated gene regulation (10, 11) Due to their inducibility by heavy metals, MTs are potential biomarkers for metal exposure in the aquatic environment [12], as well as Sulaiman [13] revealed a correlation between MT and heavy metal contents in field studies.

MTs are the sole proteins in which cadmium accumulates naturally. MTs have been proposed as biomarkers for environmental control and occupational diseases [14]. It is noteworthy by literature that methods for MT purification described thus far generally include several combined chromatographic steps, such as gel filtration, ion exchange and HPLC [15-17]. These methods take time for purification besides the high loss of protein mass along the entire procedure when compared to most procedures by affinity chromatography. Indeed, the extraction and purification of native proteins usually involve several steps unless the protein possesses some peculiar structural characteristic or physical–chemical property that allows a special procedure for purification. In that regard, MTs present a cysteine-rich primary structure. As well known, cysteine is prone to chelate transition metals. Based on that property, in the present work, we have adopted a simple method for purification.

In the present work, we have described a simple method for purification of MTs from the liver tissue of grass carp by affinity chromatography.

MATERIALS AND METHODS

The fresh water fish *Ctenopharyngodon idella* were collected from Poondi fish farm Tiruvallur district, Tamil Nadu, India. The fishes were acclimatized in the laboratory in a stone tank for 7 days at room temperature (30±20 °C). For the experiment, analytical grades, aqueous solution $CdCl_2$ were used. The concentration of (5.0 ppm) exposed for a period of 72 h.

Sample preparation

The fishes were removed from the tanks after 72 h of Cd exposure. The liver tissue was removed from the fish, handled with plastic forceps to avoid contaminations. Sampling tissues were kept in-80 ºC until further use.

MT Isolation and purification

Isolation and purification of MT was performed by following the method [18] with slight modifications.

Affinity chromatography was performed using a 1-ml Hi Trap TM Chelating HP column. The liver tissue (50 mg) was homogenized and the Supernatants were applied to the column previously loaded with $NiSO_4 \cdot 6H_2O$, respectively. After metal loading, the column was equilibrated with 10 volumes 50 mM Tris–HCl, pH 7.4 added of

0.15M NaCl, here called equilibrating buffer. After applying the supernatants, 10 volumes of equilibrating buffer were passed through the column. In extractions (liver homogenate), fractions (1 mL) were collected at a flow rate of 1 mL/min through the entire procedure. The purification was performed at room temperature. Once finished the elution procedure, the column was washed with 10 volumes of 1 mM EDTA (this step is called washing-step), followed by 20 volumes of Milli-Q fresh water to recover the column, according to the protocol enclosed to the manufacturer's technical data sheet. Aliquots (20µL) of each fraction obtained from the elution step were applied to 15% SDS–PAGE stained with CBB. MT-positive fractions were deduced on the gel by the molecular mass.

Western blot

The protein concentrations were measured using the Lowry's method [20]. The supernatant was separated and added to a new 1.5 ml centrifuge tube and stored at-20 ℃ until further use. Equal amount of total protein (50 µg) was mixed with 2X sample buffer and boiled for 5 min. The proteins were separated 15 % SDS-PAGE and electrophoretically transferred into Poly Vinylidene Di Fluoride (PVDF) membranes (Millipore, USA). The blots/non-specific binding sites were blocked with 5 % blocking buffer for 2 h. After blocking, membranes were incubated with respective primary antibodies Anti-MT antibody (ab12228-UC1MT, Abcam) 1:1000 dilution overnight at 4 ℃. Then the membranes were washed thrice with T-TBS, each for 10 min, followed incubation for 45 min at room temperature with the corresponding horseradish peroxidase-conjugated secondary antibody (HRP) (Geni, Bangalore) (1:500 dilutions). Finally, signals were visualized using super signal west femto maximum sensitivity substrate kit (Prod#34095, Thermo Scientific, USA). The signals were captured by Chemi Doc XRS system (Bio Rad, USA).

RESULTS

Isolation and purification of MT

Affinity chromatography

Metallothionein was isolated and purified from liver tissue of *C. idella* exposed to sub-lethal concentration of (5 ppm) CdCl2 for 72 h. Purification was carried out by affinity chromatography using 1 mL Hi Trap TM metal chelating column . MTs were purified by Ni2+ loaded resins and eluted with 500mM imidazol. The elution was performed with 1 ml/min and the fractions were collected. Seven eluted fractions were obtained. The highest MT concentration was obtained in the fourth fraction (fig. 1).

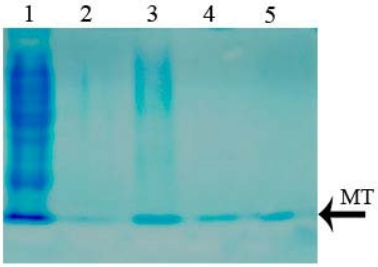

Fig. 1: Elution fractions (500 mM imidazole; flow rate 1 ml/min) of MT from liver extract started to be eluted infractions 2, 3, 4 and 5. The purest forms of MT were then visualized using 15% SDS-PAGE stained with CBB. Lane 5 shows pure MT as it had no non-specific bands. The molecular markers were helpful to identify the MT protein by comparing its approximate molecular mass (6-7 kDa)

Western blot

Purified MT protein was confirmed by Western blot with specific antibodies. The antibody shows a positive cross reactivity with MT proteins (fig. 2). Purified MT protein was detected using chemiluminescence system (ECL Kit) method. MT bands appeared in the PVDF membrane, which proves that antibody, reacted with specific MT protein after electrophoresis in 12% SDS-PAGE.

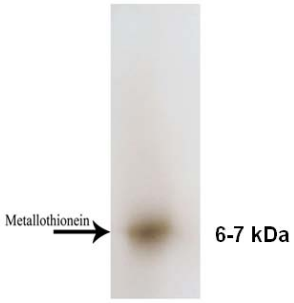

Fig. 2: Purified MT confirmed by Western blot

DISCUSSION

Cd is seen to accumulate mainly in organs such as the liver that is functionally designed to store metals and to detoxify heavy metal by synthesizing MTs (21-23). At genetic level it has been observed that MT genes are radically induced by various physiological and toxicological stimuli such as heavy metals, mitogens, cytokines, hormones, inflammation etc [24, 25].

As higher concentrations of Cd was observed in the liver tissue, it is assumed that the heavy metal Cd induces the synthesis of new MTs and synthesis and high rate of Cd-MT complex formation and the complex so formed sequesters the cadmium [26] to other detoxifying or excretory organs such as the kidneys. Cd and MTs concentrations increases significantly in fishes exposed to different concentrations of Cd for 24, 48 and 72 h in the liver tissue.

The affinity chromatography column was used in the present study, because of its accuracy in isolating the MT protein in liver tissues of *C. idella.* Sulfydryl residues are able to bind copper, zinc and cadmium with high affinity, through the formation of metal thiolate clusters [27]. Cystene in MT protein is prone to chelate transition metal such as Cd. MT isolated from the liver tissues of C. idella exclusively binds to nickel in comparison to other metals as noted by Coyle [28] MT extracted from the liver of fishes would have quite different affinity for Cd and Ni2+, which could explain why it did not bind to the Cd2+ attached to the column; however, it did bind to Ni2+, as confirmed by $Cd^{2+}$and Ni^{2+}determination from MT positive fractions. The results of the present study correlate with earlier works of Romero and Vasak [29] and Honda [18].

Affinity chromatography has been a widespread and powerful technique for purification of mainly recombinant proteins modified with specific sequences, e. g., poly histidine tails, enzymes, monoclonal antibodies, DNA-binding proteins etc. The technique is scarcely described for the isolation of native proteins unless they have any specific structural characteristic that allows a group specific adsorption. Because metallothioneins has a particular cysteine-rich structure. An option for their purification is the application of covalent affinity chromatography by thiol–disulphide interchange, as already described in the literature by Kabzinski [30, 31].

Western blot confirmed the induction and presence of MT in the tissues of the study fish. Western blot does not allow one to determine as to which specific cell-types induced MT, their localization and expression in the organs as a result of metal exposure. However, the use of antibodies against MT is accurate method to detect the presence of interested proteins (MT) [32, 33].

CONCLUSION

Affinity chromatography is a sophisticated analytical technique which has been used to isolate MT in the present study. The present study confirms that this method is best suited than other methods as stated in literature. It has been observed that over 60% of all purification techniques involve affinity chromatography. The wide applicability of this method is based on the fact that any given biomolecule that one wishes to purify usually has an inherent recognition site through which it can be bound by a natural or

artificial molecule. As Affinity chromatography is basically based on the molecular recognition of a target molecule by a molecule bound to a column, it is a very effective biochemical purification tool for isolation of low molecular weight protein. Western blot is one of the best tools to confirm the fish MT protein. Further investigations on the number and inducibility of the isoforms as well as metal binding characteristics would be needed for the use of fish MTs as biomarkers.

CONFLICT OF INTERESTS

Declared None

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