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Isolate, Purify And Characterise Metallothionein From The Hepatic Cells Of Oreochromis Niloticus [Linnaeus, 1758]

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Abstract

Objective(s): The present study intends to isolate and purify Metallothionein extracted from the liver tissue of Oreochromis niloticus exposed to Cadmium. Study of MT will help to know its protective role against Cadmium
Method(s): Acclimatised fishes were treated with sub - lethal concentration of Cadmium to induce the synthesis of Metallothionein. MT was isolated and purified from fish liver by affinity chromatography from differential distribution (3KD – 43KD) across eluted fractions. Molecular weight of the purified elute was analysed by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) and Peptide Mass Fingerprinting and confirmed to be Metallothionein.
Findings: Different elute fractions were obtained from the MT specific Affinity Column chromatography. At each fraction-by-fraction purification led to obtaining a single band in the seventh elute fraction. This single band was analysed using MALDI-TOF. Trypsin digestion produced a fragmented peptide mass spectrum. The mass spectrogram revealed a single peak, indicating purified MT protein with a molecular weight of 6140.52 Daltons (~6.2 kDa). The finding contributes valuable insights into Metallothionein structure and properties, and understanding its role in metal detoxification and stress regulating cellular processes.
Novelty: Molecular weight of the Metallothionein from liver tissue of Oreochromis niloticus was found using MALDI-TOF. This method is a simple way to isolate and purify low molecular weight protein like MT.

Keywords: Oreochromis niloticus, hepatic cells, Cadmium, Metallothionein (MT), Affinity Chromatography, Metallothionein proteins, Detoxification, Purification, MALDI-TOF

1. INTRODUCTION

Physiologically pertinent metal ions participate in diverse intracellular processes, encompassing cellular signaling, electron transport chain orchestration, redox reactions, etc. [1]. Nevertheless, at elevated concentrations, these metal ions can induce cytotoxicity. Cellular entities have evolved adaptive mechanisms

to mitigate such toxicity [2]. Presently, two principal adaptive mechanisms have been posited: (a) transmembrane transport of metals and (b) intracellular chelation. Within the purview of category (b), phytochelatins are excreted in response to metal-induced stress; conversely, metallothionein proteins (MTs) are synthesized in reaction to a spectrum of stimuli. Metallothionein proteins (MTs) were initially identified in 1957 by Margoshes and Valee [3] from equine renal cortex tissue [3]. Comprising 70-150 amino acids (M_r ~ 6-14 kDa), these proteins lack aromatic amino acids such as Trp, Tyr, Phe, etc., while containing an average of approximately 20 cysteine residues per molecule [4]. MTs are characterized by an unusually high concentration of d10 metal ions (Zn2-, Cd2-, and Cu-), forming distinctive metal thiolate clusters through the sulfur atoms of the cysteine groups constituting the protein. Thiolate groups function as both terminal and bridging metal ions [4]. Various MT isoforms exist in different organisms, exhibiting minor disparities in amino acid positions, isoelectric points, and hydrophobicity [5,6]. The presence of diverse MT isoforms, each with unique roles and functions, has captivated scientific inquiry. MTs exhibit ubiquitous expression levels and respond to various kind of stress conditions. Primarily involved in the storage, transport, and exchange of trace elements, MT proteins play a crucial role in the detoxification of heavy metals [7,8]. Additionally, MTs regulate the homeostasis of essential trace elements, including copper and zinc, detoxifying both essential and nonessential metals.

Various mammalian tissues exhibit age-dependent trace or a low level of MTs as they play a crucial role in diverse metal regulatory processes, including neoplastic cell growth, cell proliferation and differentiation, etc., [9]. Reports indicate that MTs exert regulatory control over various hormones and contribute to cell metabolism, and the multifaceted functions of MTs and their distinct isoforms remain subjects of ongoing investigation [10]. Studies have unveiled the role of MTs in mitigating oxidative stress by depleting free radicals, thereby safeguarding cells [11]. Additionally, MTs modulate the functions of zinc-mediated genes. Given their inducibility by heavy metal ions, MTs emerge as promising biomarkers for assessing metal exposure in aquatic environments [12]. Sulaiman, through field studies, established a correlation between MTs levels and heavy metal concentrations [12]. Notably, among various proteomes, MTs naturally accumulate cadmium metal. Numerous investigations have projected MTs as potential biomarkers for environmental controls, occupational disorders, and many other diseases [13]. Human exposure to combinations of genotoxic and non-genotoxic environmental chemicals associated with cancer is commonplace. Reliable biomarkers indicative of somatic stem cell mutations and subsequent mutant clone expansion can serve as valuable proxies for cancer risk assessment [13]. The introduction of acquired mutations in a selectable endogenous reporter gene, such as glucose-6-phosphate dehydrogenase (G6PD), within colonic crypt stem cells results in a phenotype change confined to the crypt [14].

Several successful attempts have been made in the past decades, where various methods were described for the purification of MT proteins. These purification methods are as follows: Affinity chromatography (AC), ion exchange (IEC), gel filtration (GFC), and High-Pressure Liquid Chromatography (HPLC) [14–16] Following these three stepwise methods of purification requires a lot of time, and about 50-60% of the protein yield gets lost during purification, but purity gets increased when compared to another affinity-based chromatographic method [16]. In fact, the extraction, isolation, and further purification of such native proteins generally involve many steps unless the pure proteins have some structural features or physiochemical characteristics that help to adapt a special procedure to obtain pure MTs.

In this context, MTs exhibit a primary structural abundance of cysteine (Cys) residues, and these cysteine residues have a high propensity to chelate with transition metal ions [17]. Leveraging this characteristic, our current work utilizes a simplified purification method [16]. In the present study, we have isolated, purified, and determined the molecular weight of the metallothionein protein extracted from the liver tissue of Nile tilapia (*Oreochromis niloticus)*. The Nile tilapia (*Oreochromis niloticus*), a prominent fish species in aquaculture, holds the distinction of being one of the most extensively farmed fishes globally. It plays a pivotal role in enhancing local livelihoods, particularly in developing nations. The initial cultivation of O. niloticus occurred in Kenya in 1924. On an annual basis, O. niloticus contributes significantly, representing approximately 60% (approximately 15,000 tonnes) of the total aquaculture production [18–20].

2. MATERIALS AND METHODS

The Nile tilapia (*Oreochromis niloticus)* was obtained from Pulicat Lake (13.4339°N, 80.3214°N) of Tiruvallur district, Tamil Nadu, India. They were maintained in the laboratory in a stone tank (100 litres) for a week at room temperature (RT) (30±20 °C). To carry out further experiments, an analytical grade aqueous solution of cadmium chloride ($CdCl_2$) was used in the study. The fishes were exposed to a 5.0 ppm concentration of $CdCl_2$ for a period of 96 h.

2.1 Sample preparation

The specimens used in the study were exposed to Cd for 96 hours. Later, the fish were taken out of the tank, anesthetized using MS-222 and liver tissues was extracted using plastic forceps to mitigate the risk of contamination. Afterward, tissues samples were preserved at -80 °C for subsequent utilization.

2.2Cadmium accumulation in the liver tissue of Oreochromis niloticus

To ascertain the level of Cadmium in liver tissue of the *Oreochromis niloticus*, the isolated liver tissues were placed on petri plates and desiccated in an oven (ILE co, Chennai, India) at 110 °C for 48 hours. The tissue samples were digested using a mixture of nitric acid (HNO3) and perchloric acid (HClO4) in a 2:1 ratio on a hotplate (ILE co, Chennai, India) at 100 °C for 5 hours. Once the digestion was completed, 5 ml of distilled water was added to each sample, and the concentrations of cadmium (Cd^{+2}) was determined by the Atomic Absorption Spectrophotometer (Perkin Elmer AAS GHA 900, USA).

2.3 Total tissue Protein estimation

5 g liver tissue was homogenized using a glass homogenizer (Airblow, Chennai, India) in lysis buffer containing 100 mM phosphate buffer (pH 7.4), supplemented with 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 0.5 mM Dithiothreitol (DTT), and 1% NaCl. After lysis, the homogenate was centrifuged at 10,500 rpm for 30 minutes at 4°C, and the resultant supernatant was collected for further analysis. The protein content was determined using the protein-dye binding method in the tissue culture samples, as per Bradford's method [21].

2.4 MT quantification

The liver tissue of the fish was saturated with Cadmium Chloride in vitro at the concentration of 6.4mg/kg (Onosaka et al., 1984). The excess Cadmium and all the Cd ligands other than MT in the sample was removed by the addition of RBS hemolysate in a subsequent heating step. Since it is known that 1 mole of thionein (MW 6050) binds with 6-7 atoms of Cd, the actual concentration of MT in the liver tissue is calculated after estimation of Cd^{2+} in the heated supernatant using AAS as presented above.

2.5 MT isolation and purification

Metallothionein was isolated and purified as per standard methods, with minor modifications. Affinity chromatography (AC) was employed with a 1-ml HiTrap™ Chelating HP column. 50 mg liver tissue was homogenized in a homogenizer while keeping the sample in ice. After that, the samples were centrifuged at 12000 rpm for 30 min at 4 ^{0}C. The supernatant was collected and loaded onto the nickel-charged HiTrap HP column, which was pre-equilibrated with 10-column volumes of an equilibration buffer (50 mM Tris–HCl, pH 7.4, 0.15M NaCl). Subsequently, the column was washed to a 10-column volume with a wash buffer. After washing the column, the protein was eluted using an elution buffer with fractions (1 mL each) collected at a flow rate of 1 mL/min. The entire purification procedure was conducted at 4 ^{0}C to avoid any misfolding of the protein. At the end of the elution, the HiTrap ™ Chelating HP column underwent thorough washing with 20 mL of washing buffer supplemented with 1 mM EDTA, followed by 20 mL of autoclaved Milli-Q water to regenerate the column, in accordance with the instructions provided by the manufacturer's technical data sheet. After purification, a 9 µL aliquot from each eluted fraction with 3 µL SDS-loading dye (4X) was applied to an 15% SDS–PAGE. The gel was stained with Coomassie Blue (CBB) and further destained. The molecular weight (M.W.) of MT was qualitatively analysed through gel electrophoresis.

3. MALDI-TOF

The purified proteins were resolved on the SDS-PAGE (15%) and further visualized by CBB R-250. For proteomic analysis, the protein bands were excised from the gel, sliced into 1 mm² cubes, and incubated overnight at room temperature with 200 µL of 50 mM NH_4HCO_3 in 40% ethanol. Once the stain was removed, the liquid was taken out and replaced with 50 µL of 50 mM NH_4HCO_3. Next, the NH_4HCO_3 buffer was replaced with 150 µL of acetonitrile and incubated for 10 min, and this step was repeated two times. The gel pieces were dried for a few minutes, and twenty microlitres of fresh trypsin solution (12.5 ng/µL) in 50 mM NH_4HCO_3 buffer was added to the tube on ice. The excess enzyme solution was discarded using the gel loading tips after 10 min of rehydration, and the solution was replaced with an equivalent volume of fresh 25 mM NH_4HCO3 buffer, followed by incubation for 12 hrs at 36°C. The digestion was terminated by the addition of 10 µL of 1% trifluoroacetic acid (TFA) for MALDI-TOF MS analysis. Without any trypsin digestion, the purified

protein is also taken for the MALDI-TOF MS for molecular weight determination. After that, the 20 µL of trypsin digested purified protein or pure protein solution was used for the MALDI-TOF MS analysis, recorded on an AB SCIEX Voyager DE Pro MALDI-TOF (Applied Biosystems, Foster City, CA) time-of-flight spectrometer, with a pulsed nitrogen laser (337 nm; 3-ns pulse width). The spectra were recorded in the linear, positive, high-mass mode. A saturated solution of a-cyano-4-hydroxycinnamic acid in a 1:1 mixture of acetone and water and 0.1% trifluoroacetic acid was used to obtain the mass spectra. The products of trypsin digest will be analysed for identification of the protein fragments, the Mascot program (http://www.matrixscience.com) was utilized for selected peptide masses against SwissProt databases search. The highest protein score, sequence coverage, similar molecular weight, and related species were considered for the identification [22,23].

4. RESULTS

Quantification of Cadmium in liver tissue of *Oreochromis niloticus*

Atomic Absorption Spectroscopy (AAS) was used to quantify Cadmium accumulated in the liver tissues of Oreochromis *niloticus* and found to be **0.49mg/kg.**

4.1Total Protein estimation

The protein extract was obtained through isolation from the liver homogenate, yielding concentrations of 209.64 mg/kg and 279.29 mg/kg in the control and treated fish liver, respectively. This variation is attributed to the influence of heavy metal stress, prompting the synthesis of diverse stress-response proteins and enzymes. Upon dialysis, the protein content was determined to be notably elevated at **251.43 mg/mL**. Our findings suggest a higher abundance of novel proteins in the treated sample compared to the control liver protein sample. The treated samples exhibited similar protein bands, with some proteins displaying reduced expression.

4.2MT quantification

The liver tissue after exposure to 5ppm of Cadmium for 96 hours were analysed for MT. this MT levels were estimated to be **311 µM.**

4.3 Purification of MT from *Oreochromis niloticus*

The isolation and purification of MT protein were executed using sophisticated techniques, notably employing affinity chromatography with a HiTrap™ metal chelating column (1 mL volume). This column facilitated selective binding for the purification of MT proteins. Ni2+-loaded resins were then employed to capture the MT protein, followed by elution using an elution buffer to release the bound MT protein effectively. A flow rate of 1 mL per minute was maintained to ensure efficiency and accuracy in MT protein isolation. A gradient elution was applied to elute the CdCl2-treated and untreated MT proteins, subsequently analyzed via SDS-PAGE **(Figure 1A &1B)**. Six fractions were collected from the elution, each containing varying concentrations of purified MT protein. Notably, the highest concentrations were observed in fractions C and D, as illustrated in **Figure 1B** of the experimental data. Fractions C and D exhibited the most substantial concentrations of MT protein, suggesting potential specific binding or affinity of the protein to the purification materials or the impact of cadmium exposure on its concentration within fish liver tissue.

4.4Molecular Weight Determination via MALDI-TOF MS and Peptide mass fingerprinting

Molecular weight determination of the Metallothionein (MT) protein was accomplished through Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) and Peptide Mass Fingerprinting. The MALDI-TOF mass spectrum revealed a pure MT protein molecular weight of 6140.52 Daltons (~6.2 kDa) **(Figure 2)**. To identify MT, trypsin digestion was performed, yielding a fragmented peptide mass spectrum obtained using a MALDI-TOF mass spectrophotometer **(Figure 3)**.

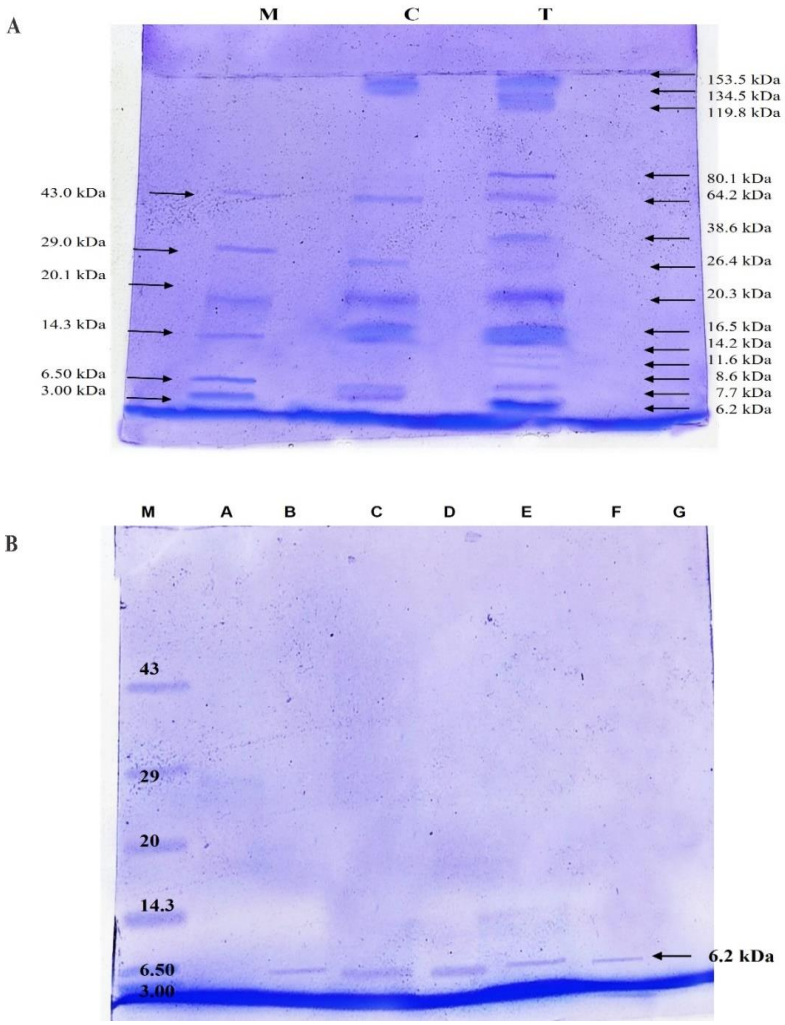

Figure 1. Expression level of proteins. (A) SDS-PAGE of isolated proteins with $CdCl_2$ treated and untreated (B) Eluted protein fractions of the purified MT protein.

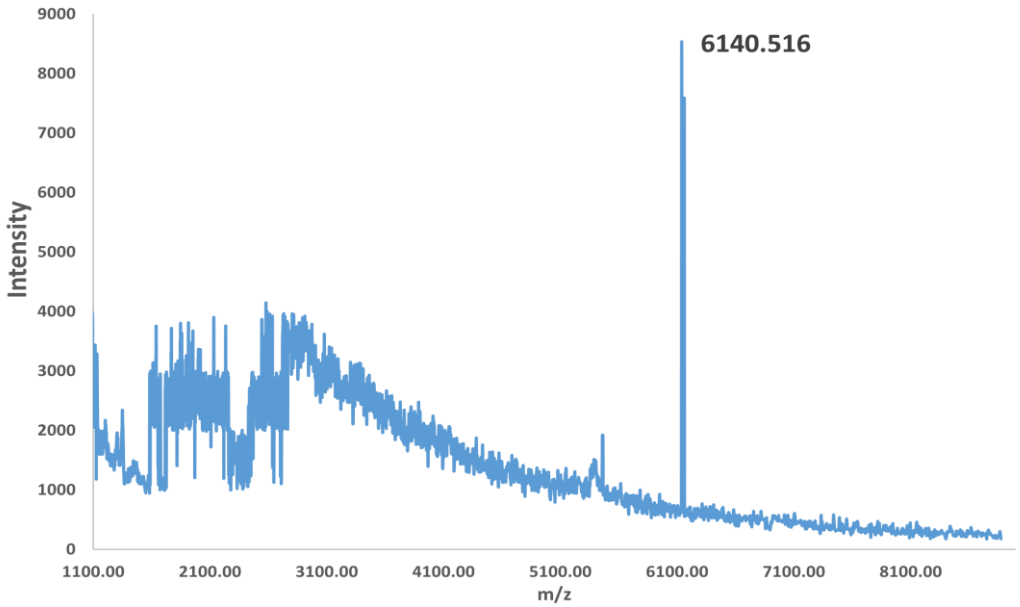

Figure 2. Molecular weight determination of MT protein using MALDI-TOF-Mass spectrum. MALDI-TOF mass spectrum showed a pure MT protein molecular weight of 6140.52 Daltons.

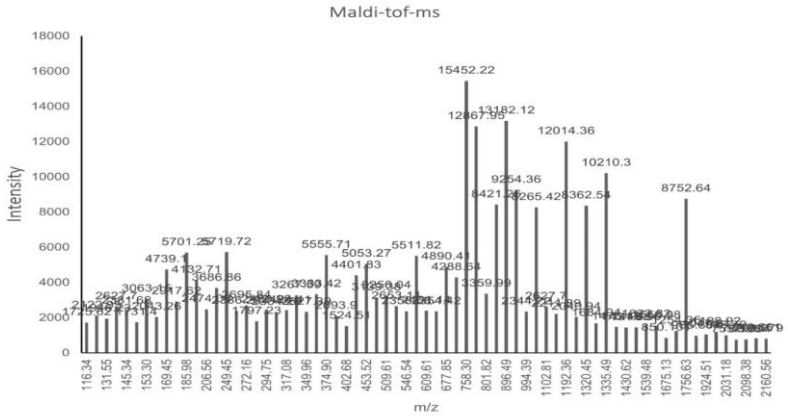

Figure 3. Peptide mass fragment from MT protein trypsin digest using Maldi-Tof-Mass spectrum.

5. DISCUSSION

Metallothionein exist in various isoforms with slight differences in amino acid positions, isoelectric points, and hydrophobicity, intriguing scientists about their diverse roles. Ubiquitously expressed, MTs respond to stress and primarily function in storing, transporting, and detoxifying trace elements, including heavy metals, regulating essential trace elements like copper and zinc while detoxifying both essential and nonessential metals [24,25] in various tissues.

In hepatic cells we found high accumulation of cadmium and enhanced synthesis of metallothionein. Accumulation of Cd (d10 group metal) was primarily observed in organs such as the liver, designed for metal storage and detoxification through metallothionein (MT) synthesis [26]. The genetic response reveals radical induction of MT genes triggered by various stimuli like heavy metals, mitogens, cytokines, hormones, and inflammation. Higher Cd concentrations in the liver suggest that Cd induces new MT synthesis, resulting in increased Cd-MT complex formation, sequestering cadmium to detoxifying organs like the kidneys. The optimum concentration of Cadmium was found to be five ppm (O.niloticus) to induce the synthesis of MT to a greater extent, indicating heavy metal toxicity protein [27].

The results of our study present assessment of heavy metal accumulation in the hepatic cells tissues of *Oreochromis niloticus*, shedding light on the intricate interplay between heavy metal stress and protein responses within the fish liver. Atomic Absorption Spectroscopy (AAS) revealed concentrations of Cadmium (Cd) at 0.49 mg/kg, respectively, in the fish liver samples. These findings underscore the environmental impact of heavy metal accumulation in aquatic organisms, emphasizing the need for continued monitoring and understanding of potential health implications for both the aquatic ecosystem and human consumers [27]. The subsequent exploration of protein extraction and estimation from the fish liver homogenate unveiled intriguing variations in protein concentrations between the control and treated groups. The elevated protein content in the treated fish liver, particularly after dialysis, suggests a dynamic response to heavy metal stress, inducing the synthesis of established stress-response proteins and enzymes[28]. The observed differences in protein expression patterns between the treated and control samples, as depicted in Figure 2A, further highlight the intricate cellular responses to heavy metal exposure [29]. Furthermore, our investigation into the isolation and purification of Metallothionein (MT) from *Oreochromis niloticus* exposed to Cadmium Chloride ($CdCl_2$) provided valuable insights into the molecular mechanisms underlying heavy metal detoxification [12,28,30]. The use of sophisticated techniques, including affinity chromatography with a HiTrap™ metal chelating column, facilitated the successful isolation of MT proteins. The differential distribution of MT protein across eluted fractions, with notably higher concentrations in fractions C and D, suggests specific binding or affinity to purification materials, potentially influenced by cadmium exposure (Figure 2B). These findings contribute to our understanding of the intricate relationship between heavy metal exposure, protein responses, and the isolation of key proteins involved in detoxification processes. The identification of MT protein and its distribution across fractions provides a foundation for future studies exploring the functional significance of these proteins in the context of heavy metal stress. Additionally, our results underscore the importance of employing advanced analytical techniques, such as AAS, SDS-PAGE, and affinity chromatography, in unraveling the complexities of molecular responses to environmental stressors in aquatic organisms.

The molecular weight determination of the Metallothionein (MT) protein through Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) [31,32] and Peptide Mass Fingerprinting [33] has provided valuable insights into the structural characteristics and identification of MT

protein. The MALDI-TOF mass spectrum unveiled a precise molecular weight of 6140.52 Daltons (~6.2 kDa) for the pure MT protein.

6. CONCLUSION

In conclusion, our study has provided valuable insights into the cellular responses of *Oreochromis niloticus* to cadmium exposure by the synthesis of metallothionein (MT) protein, a key player in heavy metal detoxification. Through a systematic investigation, we determined that the optimal concentration of cadmium inducing the synthesis of MT to a greater extent is five parts per million (ppm), underscoring the significance of this heavy metal as a trigger for the expression of MT, a crucial protein in heavy metal toxicity response. The purification of MT protein was achieved through the application of advanced analytical techniques, notably affinity chromatography and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF). These methods allowed for the isolation and purification of MT protein, providing a clear understanding of its molecular characteristics. The molecular weight determination revealed a purified MT protein with a molecular weight of approximately 6 kilodaltons (kDa), which aligns with previous findings in the literature. This study enhances our comprehension of the intricate cellular mechanisms activated in response to heavy metal stress in aquatic organisms, emphasizing the pivotal role of Metallothionein as a protective protein. The identified optimal concentration of cadmium for inducing MT synthesis provides practical information for future studies and environmental monitoring efforts. Additionally, the successful application of affinity chromatography and MALDI-TOF in the purification and characterization of MT protein underscores the efficacy of these analytical methods in proteomic research. Overall, our findings not only contribute to the broader knowledge of heavy metal detoxification mechanisms but also demonstrate the applicability of sophisticated analytical techniques in unravelling the complexities of protein responses to environmental stressors. This knowledge is crucial for understanding and managing the impact of heavy metal contamination in aquatic ecosystems.

CONFLICT OF INTERESTS

Declared None

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