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Characterization of metallothionein protein from hepatopancreas organ of *Pilsbryoconcha exilis* collected from Cikaniki River, Western Java, Indonesia

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Abstract. *Rahayu SYS, Prihatini W. 2020. Characterization of metallothionein protein from hepatopancreas organ of Pilsbryoconcha exilis collected from Cikaniki River, Western Java, Indonesia. Nusantara Bioscience 12: 1-5.* Freshwater environment, undergoing various changes due to the presence of dangerous toxic anthropogenic waste. It causes pressure on the freshwater biota that lives in it, such as *Pilsbryoconcha exilis* mussel at the bottom of freshwater. This pressure is controlled by the body through the synthesis of a set of stress proteins. Endogenous proteins, metallothionein (MT), in the body of freshwater biota absorb heavy metals in the body of biota, in the form of stress control. This research identified MT protein on *P. exilis* from contaminated waters such as the Cikaniki river with the average of mercury levels in water, sediment, and hepatopancreas of mussels using AAS method were 0.001 mg/L, 0.120 mg/L, and 1.318 mg/L respectively. Hepatopancreas of *P. exilis* was extracted using a Tissue Extraction Reagent I kit (Invitrogen), with procedures following the factory manual. The extract was purified by filtration using Sephadec 50; then, the filtration results were migrated together with the PageRuler TM Unstained Low Range Protein Ladder (Fermentas) in Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE) gel medium on Biorad Protein II electrophoresis. After completion of electrophoresis, the gel was stained using Page Blue Protein Staining Solution (Fermentas), following the factory manual procedure. Characterization at this research has succeeded in obtaining the MT-I isoform protein measuring 5, 10, and 25 kDa from the hepatopancreas organ of *P. exilis*.

Keywords: Cikaniki River, heavy metal, MT protein, *Pilsbryoconcha exilis*

INTRODUCTION

Metallothionein (MT) proteins are found in various body tissues from various species of organisms, and are easily induced by multiple stimuli. MT protein contains a lot of sulfur, is able to bind to heavy metals, have a small molecular weight (4-10 kDa), contain 26-33% cysteine (cyst) is capable of tying up and build-up of metal ions with covalent bonding, as well as not having an aromatic amino acid or histidine (Baird et al. 2006; Gagné et al. 2007). MT protein plays an important role in the detoxification of heavy metals (Viarengo et al. 1999). The detoxification process initiated by the binding of metal ions on the surface of cells, which occurs because the positive ions are bound to the side of the reactive extracellular polymer negative charge (such as R-Coo-and PO4). The next stage in the detoxification of heavy metals is the transport of metal ions into the cytoplasm, and accumulated by the MT proteins (Bernal-Hernandez et al. 2010).

The mechanism of protection against the impact of heavy metals by MT, influenced the degree of exposure to the metal to the network, but the response of MT is not the only physiological mechanism of resistance to heavy metals at home (Bernal-Hernandez et al. 2010; Amiard et al. 2008; Metian et al. 2008). At low concentrations of metal exposure, MT binding metals essential for maintaining homeostasis in the cell. On exposure to high concentrations (e.g., approaching the level of toxic and lethal), a metal-binding protein of MT with a high molecular weight and concentration of MT is known to correlate with the concentration of metals in tissues of exposed (Gagné et al. 2007).

MT affinity towards heavy metals varies; Mercury has the strongest affinity than other metals such as copper, cadmium, silver, and zinc. With its ability to bind essential metals and nonessential, MT restricts distribution of heavy metals to places that are not desired, and provide protection against metal toxicity (Amiard et al. 2008). MT known set of three fundamental processes, i.e., (i) the release of gas intermediaries such as hydroxyl radical nitric oxide or, (ii) turn off the cells that no longer function (*apoptosis*), and (iii) binding and metal exchange. The accumulation of the MT at the cellular level is influenced by, among others, gene expression, and protein degradation (Jenny et al. 2004). In humans, there are four groups (isoform) MT with specific functions (Table 1).

Table 1. Grouping of metallothionein proteins (MT)*

	MT-I and MT-II	MT-III	MT-IV
Distribution	All body cells, especially the intestinal mucosa.	Specifically, in the brain, pancreas, and intestinal cells.	Specifically, in the skin, tongue, and upper digestive epithelial cells.
Amount of amino acids	61 (20 cyst)	68 (20 cyst); 4 Cu and 3 Zn	62 (20 cyst)
Protein size	3-30 kDa		
Function	• Zn and Cu regulation • Transcription • Metal detoxification • Immune system • Digestive process	• Neuron growth inhibition • Development, organization, apoptosis of brain cell	• Regulation of stomach acidity • Sense of taste • Sun protection

Note: * in humans (Roesijadi 1994; Baird et al. 2006)

Mt-I and MT-II very easily induced by heavy metals, hormones, inflammation, acute stress, and various chemicals (Lee and Koh 2010). MT-protein I and MT-II can be found on many species of plants, animals (both vertebrate and a vertebrate), as well as human (Roesijadi 1994; Baird et al. 2006; Boateng et al. 2010). Although MT function appropriately continued investigated, although the evidence shows that MT regulates or controls the ability of the intracellular, both to the essential metals such as Cu and Zn, as well as nonessential such as metals Hg, and Pb. MT can donate Cu and Zn to the appropriate receptors (e.g., metalloenzymes and transcription factors), so that it can control the metabolic activity through very specific molecular interactions (Jenny et al. 2004).

The utilization of MT as biomarkers is based on the fact that the metals can be absorbed in the tissues of the body because of the presence of MT. The number of amino acid cyst causes the MT protein has a large amount of 'thiols' (sulfhydryl,-SH). This group binds heavy metals with very strongly and efficiently. The residue of cyst-SH can bind to metals; one ion of metal (e.g. Cd, Zn, Hg) has three residues of cyst-SH or a single metal ion with two residues of cyst-SH. (Baird et al. 2006; Lee and Koh 2010). MT binds very strongly with metal, but can also be easily exchanged with other proteins, because MT-metals bonds have high thermodynamic stability, but their kinetic stability is low. Therefore, biologically, MT can function as a distributor and intracellular mediator that it binds (Ryvolova et al. 2011; Shutkova et al. 2012).

Increased concentrations of heavy metal in the human body stimulate the formation of free radicals, and reactive oxygen species (ROS), resulting in oxidative damage. This condition is responded by the body by synthesizing proteins and MT which acts as an antioxidant. Intracellular MT acts as a repository of essential metals, scavenger of ROS, and regulatory activity of transcription factors. MT in extra cells plays a vital role in the regulation of various cell functions, for example, the development of adaptive immunity function (Kusakabe et al. 2008; Lee and Koh 2010; Gupta and Singh 2011).

The purpose of the research was to characterize MT protein from hepatopancreas organ of *Pilsbryoconcha exilis* collected from heavy metal contaminated waters.

MATERIALS AND METHODS

Study area

Local freshwater mussels samples of *Pislbryoconcha exilis* were collected from the Cikaniki river, Bogor Regency, West Java Province (Figure 1). The distribution of mercury in the soil, sediment, and stream water around the artisanal small-scale gold mining area along the Cikaniki River, West Java, Indonesia, was investigated (Tomiyasu et al. 2013). Research results showed that mining waste could be transported with the river flow and deposited along the river. The slope of the line was more significant near the village, implying a higher rate of deposition of mercury. The T-Hg in the sediment ranged from 10 to 70 mg kg^{-1}, decreasing gradually toward the lower reaches of the river.

Procedures

Collection of Pilsbryoconcha exilis *freshwater mussel*

Samples of freshwater mussels were divided into two groups consisting of three mussels; Hepatopancreas of the first group was taken for MT protein analysis. The second group was used as a reservation.

Analysis of mercury levels in sediment, mussels and river water of Cikaniki

The mercury concentrations in the sediment, mussels, and river water of Cikaniki using Atomic Absorption Spectroscopy (AAS) method has been carried out in the Biology Laboratory, Universitas Pakuan, Bogor, Indonesia while the characterization of MT protein has been carried out in the Molecular Genetic Laboratory, Universitas Pakuan, Bogor, Indonesia.

Data analysis

Characterization of MT proteins

0.1 mg of the hepatopancreas of *P. exilis* was extracted using a Tissue Extraction Reagent I kit (Invitrogen), with procedures following the factory manual. The extract was purified by filtration using Sephadec 50; then, the filtration results were migrated together with the PageRuler TM Unstained Low Range Protein Ladder (Fermentas) in Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE) gel medium on Biorad Protein II

electrophoresis. Gel levels were 5% and 15% level gels with gel composition referring to Shagger (2006). Tris-Tricine-SDS pH of 8.25 was used as a gell buffer; while Tris HCl pH 8.9 was used for electrophoresis buffer. After completion of electrophoresis, the gel was stained using Page Blue Protein Staining Solution (Fermentas), following the factory manual procedure. The stained gel was then observed under the light of the gel reader lamp.

RESULTS AND DISCUSSION

Analysis of mercury levels in sediment, mussels and river water of Cikaniki

The results revealed that the average of mercury levels in water and sediment were 0.001 mg/L and 0.120 mg/L which below the minimum threshold. However, hepatopancreas samples from mussels at the observation station were contaminated with mercury with average value above the threshold of 1.318 mg/L (Table 2).

The size of the protein metallothionein (MT) Pilsbryoconcha exilis

The results of SDS-PAGE electrophoresis showed various sizes of MT proteins in *P. exilis* hepatopancreas tissue which were five kDa; 10 kDa; and 25 kDa (Figure 2). There was also a large protein (> 30 kDa), which were considered as protein group related to stress control, namely heat shock protein (Hsp).

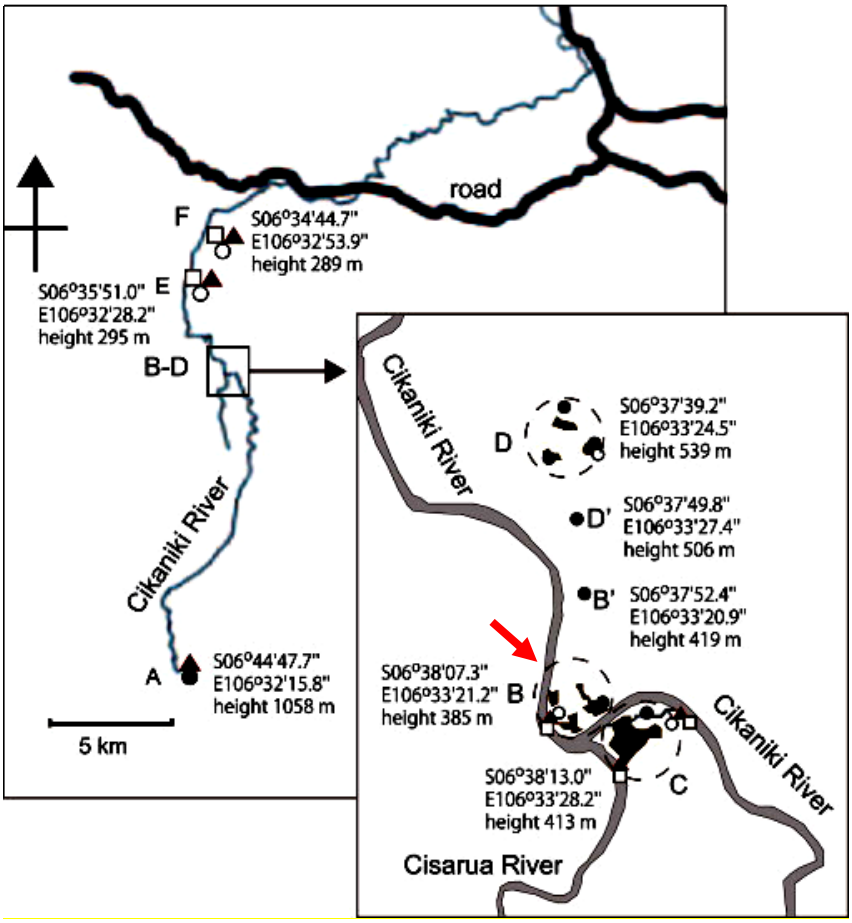

Figure 1. The sampling sites of *Pilsbryoconcha exilis* in Cikaniki river: red arrow (506º38'07.3 E1106º32'21.2" height 385 m) and the detected sites (source: modified from Takashi Tomiyasu et al. 2013).

Table 2. Mercury levels in water, sediment, and hepatopancreas of mussels

Section (mg/L)	Mercury concentration	Threshold	Source
Water river	0.001	0.002	Government Regulation of Republic of Indonesia No. 82 (PP RI 2001)
Sediment river	0.120	0.17	Canadian Council of Ministers of The Environment (CCME 1999)
Hepatopancreas of *P. exilis*	1.318	1.0	Indonesian National Standards (SNI 7387: 2009)

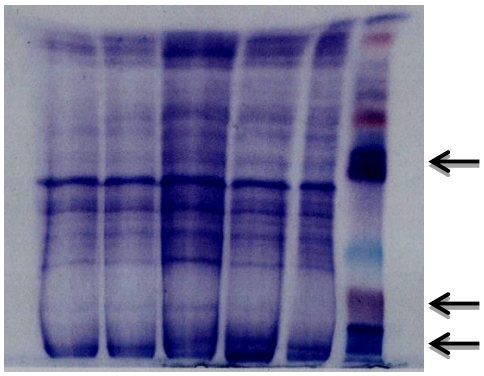

Figure 2. Size of MT protein from *Pilsbryoconcha exilis* hepatopancreas

Discussion

We obtained 5 MT-I protein with a molecular weight of 5; 10; and 25 kDa from hepatopancreas tissue of *P. exilis* collected from the Cikaniki river, and some large-sized proteins (Figure 2.), which were considered as protein group related to stress control, namely heat shock protein (Hsp). The presence of MT protein showed that *P. exilis* provides a biological response to increased concentration and duration of mercury exposure. Lee and Koh (2010) stated that under conditions of oxidative stress due to mercury accumulation, MT functions as an antioxidant in the non-enzymatic antioxidant defense system.

MT protein animals have four isoforms, namely MT-I, MT-II, MT-III and MT-IV. The isoform of MT-I and MT-II are distributed in various tissues, especially the liver, pancreas, intestine, and kidney. MT-III and MT-IV proteins are found mainly in the brain and skin of vertebrates (Amiard et al. 2006). In this study, 5 MT protein bands were successfully obtained; MT with molecular weights of 10 and 25 kDa are MT-I and MT II isoforms (Roesijadi 1994). Protein isoforms of MT-I and MT II are very easily induced by heavy metals, hormones, inflammation, acute stress, and various chemicals, and found in various animal species, both invertebrates, and vertebrates. MT affects the level of tolerance and hepatotoxicity of heavy metals, also acts as a free radicals scavengers that protect against oxidative damage (Lee and Koh 2010; Machreki-Ajmi and Hamza-Chaffai 2008).

Body tissues that are directly involved in heavy metal extraction, storage, and excretion, has a large capacity to synthesize MT proteins. In mollusks and crustaceans, generally, MT proteins are found in hepatopancreas and gills; specifically in bivalves, it is known that MT protein concentrations are higher in hepatopancreas organs than gills (Amiard et al. 2008). Biological characteristics also affect MT protein synthesis. Previous study showed that MT synthesis was reported in the body fluid of blood cockles (*Anadara granosa*) induced by Cd (Chan et al. 2002), in hepatopancreas mussels *Mytilus galloprovincialis* MT gene induced Pb (Pavicic et al. 1993), and in the gill of *Mytilus edulis* blue shells induced by Hg (Roesijadi et al. 1988). The result of this study is consistent with the results of previous studies. Local mussels *Pilsbryoconcha exilis* collected from Cikaniki river waters which are heavily polluted by Cd, Pb, and Hg metals (research results in the field), were strongly suspected of having adapted to oxidative stress by synthesizing MT protein as a form of biological response. The selection of hepatopancreatic organs analyzed in this study is in accordance with the expected goals, because bivalves are known to synthesize more MT proteins in the hepatopancreas than in the gills (Amiard et al. 2008).

Synthesis of MT protein is not the only physiological mechanism of resistance to heavy metal accumulation, on bivalve (Bernal-Hernandez et al. 2010; Amiard et al. 2008). There are several other proteins that also function as a cellular stress response (CSR) in bivalves, including heat shock protein (Hsp) and cytokines (Baird et al. 2006). It is consistent with the results obtained in this study; there were protein bands with a large molecular weight (> 50 kDa), which is considered as Hsp protein. The study by Butet (2013), showed the expression of the Hsp 70 gene (for the synthesis of 70 kDa Hsp protein), by the induction of mercury in *Anadara granosa* blood clams. Physiological changes in the shells in the process of detoxification can be observed from an increase in filtering, respiration, eating activity, growth, and reproduction, also changes in metabolism and biochemistry (Amiard et al. 2008). The detoxification process is carried out by MT protein by binding to metal ions, then accumulated into a formless granule (called brown vesicle) in a non-toxic way (Zaroogian and Yevich 1994). Observation of the histological structure due to heavy metal accumulation is important to be done before the onset of irreversible effects on animals.

Pilsbryoconcha exilis mussels from Cikaniki river waters that contaminated with heavy metals have synthesized a lot of MT protein. It is indicated by the emergence of 5 kDa, 10 kDa, and 25 kDa protein bands, which are included in the MT-I protein isoform.

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