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Identification of metallothionein protein in *Anodonta woodiana* as a biomarker of mercury (Hg) contamination

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Abstract. *Rahayu SYS, Fadila A, Fahmi MR. 2023. Identification of metallothionein protein in* Anodonta woodiana *as a biomarker of mercury (Hg) contamination. Nusantara Bioscience 15: 90-94.* Heavy metal contamination can affect the survival of aquatic biota and will accumulate in the bodies of organisms. Moreover, contamination identification at the molecular level can be analyzed using biomarker analysis. Biomarkers are responses measured individually, ranging from enzymes and xenobiotic measurements to organ and overall conditions. Biomarker analysis can be done by checking the metallothionein protein, this expression can be induced by Reactive Oxygen Species (ROS). Metallothionein (MT) has a thiol group with nucleophilic properties. As a result, this group can make Metallothionein able to find metals and free radicals. Therefore, prevention that can be done to reduce contamination at a higher trophic level requires monitoring the molecular level by observing the metallothionein protein. For example, *Anodonta woodiana* (Rea, 1834) or *kijing taiwan* induced by $HgCl_2$ aims to characterize their absorption ability in the environment through metallothionein protein. That was conducted by the SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis*)* method. SDS-PAGE electrophoresis results showed that the size of the metallothionein protein was 7 kDa, 15 kDa, and >30 kDa. Size >30 kDa is a group of proteins related to stress control or Heat shock protein (Hsp). The presence of Hsp is due to the body increasing stress protein synthesis and metallothionein to reduce normal protein synthesis. Based on the results obtained, this study revealed that *A. woodiana* could absorb $HgCl_2$, as evidenced by the metallothionein protein characterization results.

Keywords: *Anodonta woodiana*, environment, heavy metals, molecular SDS

INTRODUCTION

Heavy metals can accumulate in aquatic organisms by bioconcentration (pollutants entering directly from the water into aquatic organisms), bioaccumulation (pollutants entering through mechanisms), and biomagnification (increasing pollutant concentrations in tissues) organisms based on the food chain (Adhani and Husaini 2017). Dewi (2017), stated that mercury heavy metals (Hg) effectively bind to sulfhydryl groups (-SH) and can replace metal ions such as Zn, inhibiting organism's growth and development process, heavy metals that accumulate in aquatic organisms will generally be stored in the body, so these heavy metals will continue to be in the food chain and will cause detrimental effects. One of them is *Anodonta woodiana* (Rea, 1834) or *kijing taiwan*.

The *A. woodiana* removing metals based on a concentration gradient from high to low. Therefore, pollution prevention at a higher trophic level also requires treatment at a lower trophic level firstly, this can be done using organisms as biomarkers. Biomarker analysis could be examined Reactive Oxygen Species (ROS), which can attack various substrates in the body. For example, proteins, lipids, and nucleic acids, because this makes it possible to carry out biomarker analysis on mercury (Hg)-induced *A. woodiana.*

The *A. woodiana* species used the biomarkers in this study to view, identify, and study the uptake of heavy metals by analyzing the size of Metallothionein (MT) protein bands. The *A. woodiana* has the property of non-selective filter feeders, which allows it to filter out particles that enter the body from the water. Therefore, *A. woodiana* cannot select particles that enter the body. The *A. woodiana* belongs to the class Bivalvia which can adapt to polluted environments. That's could be caused by several factors, one of which is that heavy metals have high bioavailability, so they quickly enter the body. Some Bivalvia animals have poor metal metabolism or have the excretory capacity, soft metal, resulting in the accumulation of heavy metals in their body. The response given by aquatic organisms is by a physiological detoxification process and a molecular response that can be carried out by identifying metallothionein protein synthesis.

Metallothionein protein is a compound that can bind heavy metals. metallothionein can bind heavy metals in previous research because it contains many cysteines. In addition, metallothionein has a lot of thiol/sulfhydryl (-SH) groups. Therefore, it can bind heavy metals. Metallothionein generally increases with excessive heavy metal exposure and will increase cellular toxicity if the heavy metal entry rated exceed the metallothionein synthesis rate (Khati et al. 2012). Metallothionein protein expression can be induced by Reactive Oxygen Species (ROS), Glucocorticoids (GC), and

cytokines (Kimura et al. 2008). Preventive measures that can be taken to reduce contamination at a higher trophic level require monitoring at the molecular level by observing metallothionein proteins. This study aims to identify the protein metallothionein in *A. woodiana* by investigating the size of the associated protein band as a biomarker in mercury (Hg) contamination to preventing contamination at a higher trophic level. $HgCl_2$ induction can affect the characterization of metallothionein protein in *A. woodiana* so that it can be applied in the ground to reduce pollution by its ability to absorb heavy metals.

MATERIALS AND METHODS

Sample *Anodonta woodiana* collection

The samples of *A. woodiana* were collected using the dredges (rake) method by sweeping the bottom of the sandy and muddy waters. This was carried out at a maximum of 1 day (long day trip) for each capture by seser catching tools, namely sweeping the bottom of the waters in a straight line using active fishing gear (WWF Indonesia). As many as 36 mussels were taken with a random sampling technique. The samples were treated with different concentrations of $HgCl_2$ in 5 repetitions, while the controls were untreated. Finally, the mussel were put in a cool box the laboratory. As many as five mussels were put into each aquarium containing mercury with different concentrations (0.5 ppm, 1 ppm, 1.5 ppm).

Making Hg solution

A stock solution of Hg was made by weighing 0.13 g of Hg powder. Then the powder was dissolved in distilled water with up to 1 Liter Dilutions. This process was carried out for 3 treatments (0.5 ppm, 1 ppm, and 1.5 ppm).

Acclimatization

The collected mussels must acclimate to laboratory conditions to reduce stress on the experimental animals. Therefore before acclimatization, the clams were put into a transitional aquarium containing fresh water to clean the mussel from the mud. After which, they were transferred to the acclimatization aquarium. Acclimatization was carried out for seven days using water from their natural habitat, and this was done to prevent changes in metallothionein protein bands in mussel. The aquarium has a circulation pump, fed with fine pellets daily ad libitum (Prihatini 2013). This pellet has a composition of microalgae and phytoplankton which has the form of small granules. The pellet is given as much as one gram with a count twice daily in the morning and evening.

Metallothionein protein analysis

Anodonta woodiana *induction*

The mussels acclimatized for seven days, they were transferred to the treatment aquarium and treated with $HgCl_2$ at a concentration of 0.5, 1, 1.5 ppm, and the control five liters of water, pure from its habitat. Five mussels were used for each treatment aquarium. On the 30th day after $HgCl_2$ induction, two individuals were collected for the hepatopancreatic tissue on *A. woodiana* and the rest as reserves. This method refers to previous research by Rahayu et al. (2019).

Protein extraction

After induction with $HgCl_2$ (0.5 ppm, 1 ppm, 1.5 ppm), hepatopancreas were taken, mashed using a mortar dish, and diluted with PBS to a pH of 7.4. The mashed samples were stored in a refrigerator at 4°C. Next 1,500 µL was added to a microtube and centrifuged at 300 rpm for 15 minutes at 4°C. The supernatant taken from that centrifugation the protein supernatant. First, the protein concentration was determined by preparing a 1000 µL blank using 800 µL of distilled water and adding 200 µL bio rad reagent. Then using supernatant of *A. woodiana* sample for 1000 µL of sample and taking 2 µL of water and adding 798 µL of distilled water and 200 µL of bio rad reagent, the homogenizing and incubating for 5 minutes. After that, it could be read using a spectrophotometer with a wavelength of 95 mm to produce the protein absorbance. This method refers to Diaman's research (2016).

Metallothionein protein separation

Metallothionein protein analysis was carried out by SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) electrophoresis method. This method refers to the Laemmli method (1970) in Diaman (2016) and previous research conducted by Rahayu et al. (2019). Therefore to prepare for gel printing, the glass plate, comb, and spacer were cleaned with detergent and 70% alcohol. Once the gel printer is prepared, the separating gel made in the gel printer is inserted and waits for polymerization to occur. Then put the stacking gel above the separating gel, put a comb on it, and waiting for polymerization. The comb is slowly lifted from the top of the stacking gel. After that, it was put into the electrophoresis apparatus, and the running buffer was inserted and heated using electricity from the SDS-PAGE tool for 15 minutes. Buffer gel with Tris-Tricine-SDS pH 8.25 composition and electrophoresis buffer gel using Tris HCL solution at pH 8.9. The sample was inserted into the well by 20 mL, and given an electric current with a voltage of 40 volts (bromophenol blue). After bromophenol blue reaches the bottom of the stacking gel and a voltage of 200 volts is added, the flow is stopped after bromophenol blue reaches the bottom of the separating gel. The gel is removed from the printer slowly, and Commasie Brilliant Blue R-250 is put into a dye solution with 0.1% concentration for 30-60 minutes until the protein bands are stained. Then, a destaining solution is added, which removes the color of the gel that does not contain protein. The destaining solution was changed 3-4 times until the gel was clean. The protein's molecular weight is calculated Rf and plotted on a logarithmic graph of the Rf marker protein whose molecular weight is known.

RESULTS AND DISCUSSION

Results

Metallothionein protein characterization. Before performing metallothionein protein characterization. Mercury analysis was first performed on the hepatopancreas of *A. woodiana*. The first analysis was carried out when the mussels were just taken from the river to determine the original mercury level contained in the mussel bodies. The second analysis was carried out after the mussels were induced by mercury in the aquarium for 30 days. The second analysis was carried out to see how much mercury absorbed in mussel body and whether mussels have great potential as bioindicators in heavy metal absorption (Table 1).

From the second analysis conducted to check mercury levels in *A. woodiana*'s hepatopancreas, it is evident that the mussel can absorb mercury. After that, characterization was carried out through Metallothionein protein to see how much mercury exposure affected the size of metallothionein characterization.

Table 1. Mercury (Hg) Level in Hepatopancreas of *A. woodiana*

Date	Sample	Hg (mg/kg)
June 3, 2022	Hepatopancreas of *A. woodiana*	0.011
July 4, 2022	Hepatopancreas of *A. woodiana*	0.015

Discussion

This research was conducted of prevent pollution to a higher trophic level, because, at lower trophic levels, it has been detected that there is pollution through the biomarker method. In this study, metallothionein induction by Hg was carried out on the *A. woodiana* to observe the absorption of heavy metals that can affect metallothionein protein characterization. metallothionein analysis was conducted on mussels with Hg induced at 1.5 ppm treatment, This was done because none of the samples survived in the treatment of 0.5 ppm and 1 ppm. Several factors could affect this, such as feed deposits turning into ammonia is toxic to animals. If the metallothionein protein does not function normally or the amount of heavy metals exceeds the heavy metal binding capacity, then metallothionein also does not function normally (Prihatini 2013). The difference in tolerance of a species to heavy metal toxicity can be determined by the effect of the species-specific ability to regulate heavy metals and the bioavailability of the metal itself (Ramzy et al. 2021). This can be interpreted as the percentage and speed of the active substance in heavy metal when it reaches the body's systemic circulation; the body of the mussels responds differently. This can result in some mussels surviving and some not surviving because the response of each mussel's body is different in regulating heavy metals. Metals Pb, Cd, and Hg are toxic because they effectively bind with the enzyme system's sulfhydryl group (SH) to forming metalloenzymes and metalloproteins. As a result, the cells' enzyme activity cannot be processed. Prihatini (2013) states that in the group that can survive, mussels can respond to heavy metal content through growth dilution, namely dilution of metal concentrations in the body by increasing volume and fluid for toxic metal effects during growth in mussel. The presence of metallothionein serves to bind essential heavy metals needed by aquatic organisms, but while metallothionein binds too much metal, there will be an increase metallothionein.

This study uses the SDS-PAGE electrophoresis method (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis) to separate proteins based on the molecular weight in kDa (Kilo dalton) units. The organ used for analysis is the hepatopancreas section. Based on references from previous studies, it is stated that the hepatopancreas absorbs more metals. Therefore, the hepatopancreas, is responsible for detoxification.

The results of SDS-PAGE electrophoresis showed the size of the metallothionein protein in hepatopancreas tissue, with a size of 7 kDa and 15 kDa, and there was a large protein size of >30 kDa (Figure 1). Metallothionein generally has a size of 6-7 kDa (Dewi 2017), and in the results of this study, there were 9 metallothionein that had a value above 6-7 kDa. This indicates more heavy metal uptake than usual because the amount and duration of heavy metal exposure can influence the amount of MT. In the results of SDS-PAGE electrophoresis with a size of >30 kDa, which is considered a group of proteins related to stress control or Heat shock protein (Hsp). The presence of Hsp is due to the body increasing stress protein synthesis and metallothionein reducing normal protein synthesis in response to stress. This result is corroborated by previous research by Butet (2013), which showed protein synthesis of 70 kDa by mercury induction in blood clams (*Anadara granosa* Linnaeus 1758).

Metallothionein has a thiol or sulfhydryl group with nucleophilic properties. The presence of this group makes Metallothionein able to bind metals and free radicals (Lee and Jae 2010). Therefore the metallothionein protein can protect cells from deadly compounds with this function. Thirumoorthy et al. (2007) stated that metallothionein protein consists of MT-1 and MT-2 isoforms and is very sensitive to the presence of heavy metals in animals. Metallothionein protein expression is induced by Reactive Oxygen Species (ROS) or free radicals, glucocorticoids (GC), and several cytokines (Kimura et al. 2008). Metallothionein protein synthesis generally involves the Metal Transcription Factor-1 (MTF-1) protein (Dray et al. 2008) which is a Zn sensor and is located in the cytoplasm (Formigari et al. 2008). It is associated with functioning genes in Zn homeostatic processes. Based on Langmade et al. (2000) state that MTF-1, which is active after the accumulation of Zn, will then translocate into the nucleus, where it will bind to the Metal Response Element (MRE) contained in the promoter of the metallothionein gene, which triggers the expression of this protein. Zn^{2+} ion is an essential component in protein regulation and is included in the control of intercellular homeostasis (Smith et al. 2008). Zinc is included in the essential metals needed by 300 enzymes in biological activity, for example, if a zinc deficiency in cells will inhibit growth (Prasad and Bin 2019).

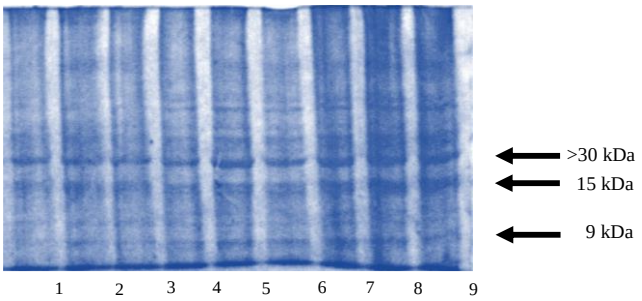

Figure 1. Results of protein characterization by SDS-PAGE electrophoresis

The metallothionein classification is based on four classes based on metallothionein location in the organ and metal type binding (i) MT-1, an metallothionein protein that has a cysteine site and is closely related to the cysteine site in mammals, for example, in mollusks, (ii) MT-2, an metallothionein protein that has nothing in common with mammalian metallothionein, (iii) MT-3, consists of non-protein metallothionein known as phytochelatin.

Metallothionein has the following protein structure: (i) Thionein, a protein with many groups, (ii) Metalation is a chemical reaction because a hydrogen atom is replaced with one of the metals. For example, in Cu, which Hg replaces, the increase occurs in the thiol/sulfhydryl (-SH) group, (iii) Dimerization is the joining of two monomer molecules to form a dimer.

When heavy metal pollution occurs in waters, aquatic organisms will experience three stages of response. The first is the shock recovery stage along with an increase in biosynthetic processes (mitosis and protein synthesis) to repair the damage and restore physiological disorders; the second stage is the damage stage and restores the disturbance. Physiologically, the production of metal-binding proteins such as metallothionein will bind at this stage. Finally, the upregulation of ions occurs to counter the damaging effects of heavy metals. Ultimately, the internal organism physiology will return to its initial state, forming a new balance. This process increases the tolerance of organisms to heavy metals (Soegianto et al. 2018).

The metallothionein method protects cells from lethal compounds, and metals enter the cells due to a passive diffusion process (metals are categorized into cells based on a high concentration gradient to low concentration). The increased concentration of heavy metals in the body stimulates the formation of Reactive Oxygen Species (ROS) free radicals that cause oxidative damage. The body will respond by synthesizing metallothionein protein which acts as an antioxidant. Heavy metals bound by metallothionein protein will be stored in amorphous intracellular granules called "chocolate vesicles" in detoxification. Therefore it will increase the number of tubular epithelial cells, hemocytes, or brown vesicles in the connective tissue. This process involves detoxification and tissue recovery (Prihatini and Mulyati 2013). Detoxification can also be observed from the filtration rate and increased feeding.

The chemical effects of heavy metals can impact the number of heavy metals that accumulate in the body of aquatic organisms. These heavy metals will generally bind to lipids and proteins in living tissue. Proteins that contain metal in their protein structure are the main protein target for heavy metal binding (Rumahlatu et al. 2012). The induction of metallothionein synthesis comes from regulating metallothionein gene expression originating from transcription and translation processes. Metallothionein genes will convey the increasing metals in cells through metal activation of transcription factors to bind certain metals (Soegianto et al. 2018). Heavy metals are known to stimulate the transcription of the metallothionein gene so that its production speed will increase. The kinetic stability of the metallothionein structure is shallow; because of that, metallothionein can bind metal very strongly (Dewi 2017). The presence of metallothionein protein in this study showed that *A. woodiana* was produced as a biological response to an increasing concentration and duration of mercury (Hg) exposure.

In conclusion, this research shows that the metallothionein protein in *A. woodiana* has sizes of 7 kDa, 15 kDa, and > 30 kDa, indicating a biological response to an increasing concentration and duration of mercury (Hg) exposure. Induction of $HgCl_2$ can affect the characterization of metallothionein protein. This research concluded that *A. woodiana* could be used as a bioindicator in an environment. This research also concluded that cleaning pollutants in polluted ecosystems is necessary.

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