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Metallothionein induction and heavy metal accumulation in white shrimp *Litopenaeus vannamei* exposed to cadmium and zinc

Jui-Pin Wu[a], Hon-Cheng Chen[b,*]

[a]*Institute of Zoology, National Taiwan University, Taipei 106, Taiwan, Republic of China*
[b]*Institute of Fisheries Sciences, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei 106, Taiwan, Republic of China*

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Abstract

Metallothioneins (MTs) have been widely considered for their potential use as specific biomarkers to reflect the existence of heavy metal pollution, because their induction has been observed to be obviously elevated after heavy metal exposure in a large number organism studied. However, relatively fewer efforts have been made in MT-related studies of prawn species, such as the white shrimp *Litopenaeus vannamei*, a globally important aquaculture species. With the results from gel filtration chromatography, we demonstrate the existence of MTs or MT-like proteins in *L.vannamei*. We further studied the relationship between MT induction and metals accumulation after long-term exposure to the heavy metals Cd and Zn. From our results, it is very clear that the response of *L. vannamei* to Cd differs from that to Zn, and this should be considered when using MTs in field applications to monitor metals contamination.

Keywords: *Litopenaeus vannamei*; Biomarker; Cadmium (Cd); Zinc (Zn); Metal accumulation; Metallothionein (MT); Invertebrate; Crustacean; Prawn

1. Introduction

Aquaculture is one aspect of commercial fisheries which provides a large portion of aquatic products to human beings, and prawn farming, highly developed in North, Central, and South America as well as in Asia, is very important among aquaculture industries. The white shrimp *Litopenaeus vannamei*, geographically distributed in Central and South America (Paez-Osuna and Ruiz-Fernandez, 1995), is a globally important prawn culture species. Traditionally, most shrimp culture farms are located near the coast, and seawater from coastal waters is directly used to rear the prawns with no additional processes. However, the coastal portion of seawater is often contaminated by many kinds of pollutants and human pathogens because of human activities (Chua, 1992; Paez-Osuna and Tron-Mayen, 1996). Therefore, there must be some risks of directly using natural coastal seawater for aquaculture, such as direct toxic effects on cultured organisms themselves, as well as adverse effects on humans. To minimize the impacts of heavy metals on ecosystems, aquaculture, and humans who consume these products, many studies have pursued the direct monitoring and detection of the distribution of heavy metals in coastal waters, bay areas, and tissues of aquatic animals (Paez-Osuna and Ruiz-Fernandez, 1995; Mantelatto et al., 1999; Turoczy et al., 2001). Additionally, some investigations have been devoted to the development and assessment of certain biological parameters as specific markers or indicators which reflect the existence or quantity of heavy metals (Kille et al., 1992; Pederson et al., 1997).

Widely defined metallothioneins (MTs) include all of the metal-thiolate polypeptides that resemble equine renal MT, occur in a large number of organisms, and possess some unique properties such as low molecular mass, metal-binding ability, cysteine richness, and heat resistance. These MTs play important roles in the regulation of essential metals and detoxification of the unusual entry of essential

* Corresponding author. Tel.: +886 2 2363 0231x3324; fax: +886 2 2363 6837.
E-mail address: honcheng@ccms.ntu.edu.tw (H.-C. Chen).

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and nonessential metals (Roesijadi, 1992). Large parts of MT-related studies have focused on the potential of using MTs as specific biomarkers of heavy-metal exposure, because induction of MT within some target tissues has been known to be clearly elevated after animals were exposed to metals (Benson et al., 1990; Roesijadi, 1992; Pederson et al., 1997).

Cadmium (Cd) is an important metal with many industrial applications. Also, cadmium is a by-product of zinc and lead mining and smelting (Klaassen, 2001). All of these applications increase the possibility for Cd to enter the environment in unusual ways and in various quantities that can cause adverse effects to ecosystems and humans. Studies have been carried out on the effects of Cd on many aquatic organisms, especially concerning the relationship with MT induction (Hidalgo et al., 1985; Stone et al., 1986; Roesijadi and Klerks, 1989; Eriksen et al., 1990; Norey et al., 1990a,b; Martinez et al., 1993; Pederson et al., 1997; Smet et al., 2001), but relatively few studies are available on prawns, which are important aquaculture species that may cause indirect adverse effects on human beings through the food chain and even spread these effects globally through commercial importation and exportation.

Zinc (Zn) is an ubiquitous and nutritionally essential metal playing a role as cofactor in more than 200 metalloenzymes and a functional component of transcription factor proteins contributing to gene expression and regulation (Klaassen, 2001). Furthermore, zinc also plays important roles in nervous and immune systems, in the optimal metabolism of vitamin A, and in normal calcification of bone. For aquatic crustaceans like *L. vannamei*, deletion of zinc supplements produces a significant depression in tissue mineralization (Davis et al., 1992; Davis and Lawrence, 1993). However, although excessive exposure of animals to zinc is relatively uncommon, it does occur around the world, especially in coastal areas, and many studies have monitored the concentration of zinc in the field (Paez-Osuna and Ruiz-Fernandez, 1995; Paez-Osuna and Tron-Mayen, 1996; Pederson et al., 1997; Mantelatto et al., 1999; Turoczy et al., 2001), as well as having investigated the acute and chronic toxicity of zinc to aquatic organisms (Vanegas et al., 1997; Zyadah and Abdel-Baky, 2000). Also, some investigations have focused on the induction of MTs or metal binding proteins after organisms were exposed to zinc (Hidalgo et al., 1985; Eriksen et al., 1990; Pederson et al., 1997; Smet et al., 2001).

Hence, the objectives of our present study were first to identify the MTs or MT-like proteins of *L. vannamei* as a foundation, then to investigate the induction of MTs and metal accumulation patterns within the tissues of *L. vannamei* under long-term exposure to Cd and Zn, and finally to further explore and discuss the practicality of using MTs as biomarkers for monitoring heavy metal pollution within prawn culture pools.

2. Materials and methods

2.1. Animals and rearing conditions

Postlarvae *L. vannamei* were purchased from a commercial shrimp hatchery in Pingtung, southern Taiwan and maintained in the laboratory for over 2 months until they reached the juvenile stage (1.39 ± 0.25 g in mass; 6.25 ± 0.38 cm in length). Water conditions during shrimp rearing and the experimental period were: temperature—25 °C, salinity—15 ppt, DO—5.8 ~ 6.5 mg/l, pH 7.15 ~ 7.87, and Eh—32 ~ 152 μS/cm, under a 12 : 12-h light–dark regime with continuous aeration.

2.2. MT identification with gel filtration chromatography

L. vannamei individuals were divided into three groups. One group was kept in clean seawater as the control, a second group was exposed to 0.2 mg Cd/l as $CdSO_4$, and a third group was exposed to 0.3 mg Zn/l as $ZnSO_4$. After 3 days, shrimp were sacrificed, and the hepatopancreas of each shrimp was dissected out. MT was identified by the procedures modified from the methods described in Wong and Rainbow (1986). Hepatopancreas samples were weighed and placed in a 1.5-mL homogenizing tube kept on ice. An approximately equal volume of homogenizing buffer was then added. In addition to Tris–HCl (0.02 M Tris, 0.01 M NaCl, and HCl added to adjust the pH to 8.6), the homogenizing buffer also contained 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and 0.1 mM dithiothreitol (DTT). The mixture was homogenized with a pellet pestle (Kontes Glass Company, Vineland, NJ, USA) and the homogenate was centrifuged at 20,000 $\times g$ for 30 min at 4 °C. The supernatant was then heat-treated in a water bath (80 ~ 90 °C for 3 min) followed by centrifugation at 1000 $\times g$ for 5 min. The steps from heat treatment to centrifugation were repeated three times. The supernatant was applied to a Sephadex G-75 (superfine) column (1.3 × 23 cm) or a Sephadex G-50 column (1.5 × 30 cm) and eluted with the same Tris–HCl buffer at a rate of 8 or 12.5 ml/h. Metal analysis and protein identification were performed after elution, for which 1.5 ml was collected in each fraction. The metal concentration within each fraction was measured using a polarized Zeeman atomic absorption spectrophotometer (Hitachi model Z-8100, Tokyo, Japan), while protein identification was performed by measuring the absorbance at 595 nm after adding Bio-Rad Protein Assay dye reagent (Bio-Rad Laboratories, Hercules, CA, USA) to each fraction. The molecular weight of MT was estimated using somatostatin, aprotinin, cytochrome C, and carbonic anhydrase as protein molecular weight standards on a Sephadex G-50 column.

2.3. MT quantification and metal concentration measurements

To perform the experiments of MT quantification and metal accumulation after long-term metal exposure, *L.*

vannamei were divided into six groups, each of which was kept within 15 l test water and contained at least 20 shrimp that were exposed to nominal concentrations of either 0.1 or 0.2 mg Cd/l as $CdSO_4$, or 0.05 or 0.2 mg Zn/l as $ZnSO_4$, and one control set for each metal as well. Each treatment was repeated three times. During all experimental periods, animals were regularly fed with commercial shrimp pellet feeds twice a day; test solutions as well as control seawater were renewed twice a week. Samples were taken on days 4, 7, 14, 21, 28, 56, and 84. On each occasion, shrimp were removed and sacrificed, and the hepatopancreas, gills, and muscles were dissected out. Samples of hepatopancreas and gills were freshly weighed and placed in a 1.5-ml homogenizing tube kept on ice. Preliminary treatments were the same as those used for MT identification described above. After homogenizing the tissue sample with Tris buffer containing PMSF and DTT, a one-half volume of the homogenate was used for estimating the MT concentration.

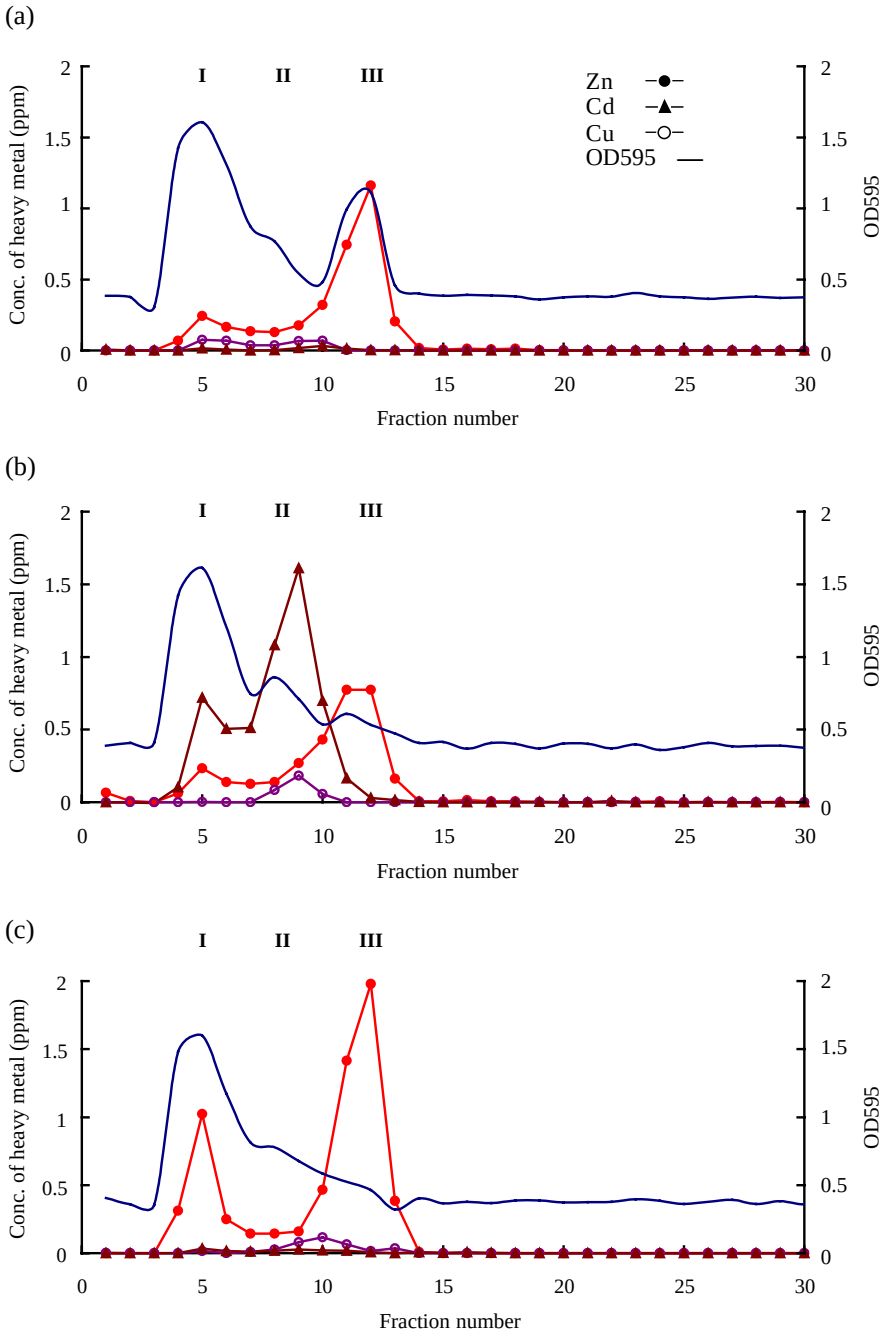

Fig. 1. Profiles of Sephadex G-75 (superfine) column elution from the hepatopancreas of (a) normal, (b) cadmium-exposed, and (c) zinc-exposed *Litopenaeus vannamei*. Flow rate, 8 ml/h. –⋆–, Zinc concentration measured within each fraction; –>–, copper concentration measured within each fraction; –▲–, cadmium concentration measured within each fraction; ǂ, absorbance at 595 nm after adding the Bio-Rad Protein Assay dye reagent to each fraction.

The method for estimating the amount of MT was based on a modified silver-saturation method (Martinez et al., 1993). Briefly, after centrifugation at 20,000 *g* for 30 min and 4 °C, aliquots of 37 μl of supernatant were incubated with 50 μl of 20 mg/l silver solution for 15 min at 20 °C to saturate the metal binding sites of MT. Following addition of 10 μl human red blood cell hemolysate, samples were heat-treated in a water bath (80 ~ 90 °C for 3 min). The denatured proteins, except for MT which is heat stable, were removed by centrifugation at 1000 *g* for 5 min. Steps from the addition of the hemolysate until centrifugation were repeated three times. The amount of silver ions in the final supernatant was proportional to the amount of MT present. The other one-half volume of the sample homogenate as well as the muscle samples were dried for 24 h at 105 °C, and then digested with 3 ml HNO_3 and 0.5 ml H_2O_2 at 95 °C to estimate the amount of cadmium and zinc within the shrimp tissue samples. Cadmium, zinc, and silver concen-

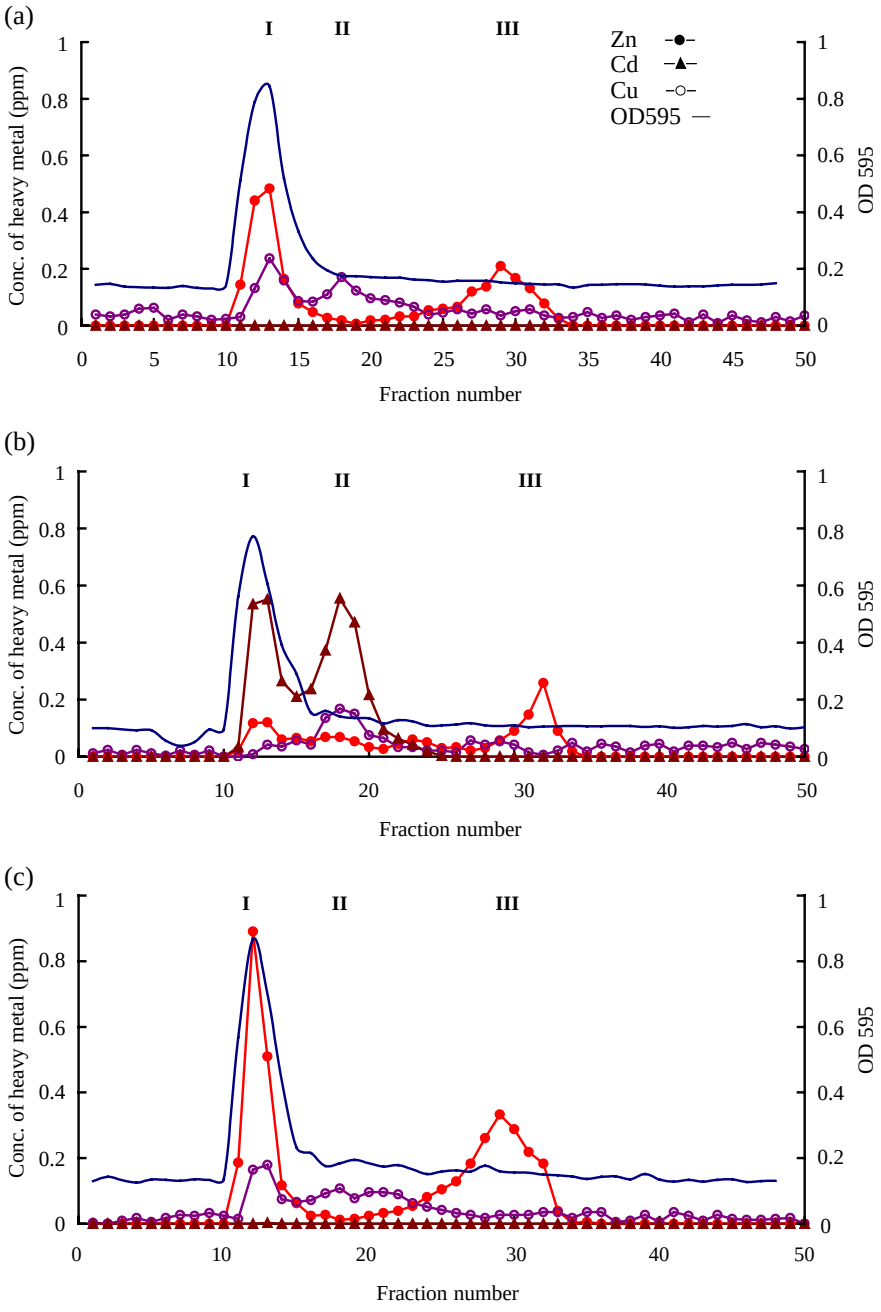

Fig. 2. Profiles of Sephadex G-50 column elution from the hepatopancreas of (a) normal, (b) cadmium-exposed, and (c) zinc-exposed *Litopenaeus vannamei*. Flow rate, 12.5 ml/h. –⋆–, Zinc concentration measured within each fraction; –>–, copper concentration measured within each fraction; –▲–, cadmium concentration measured within each fraction; ǂ, absorbance at 595 nm after adding the Bio-Rad Protein Assay dye reagent to each fraction.

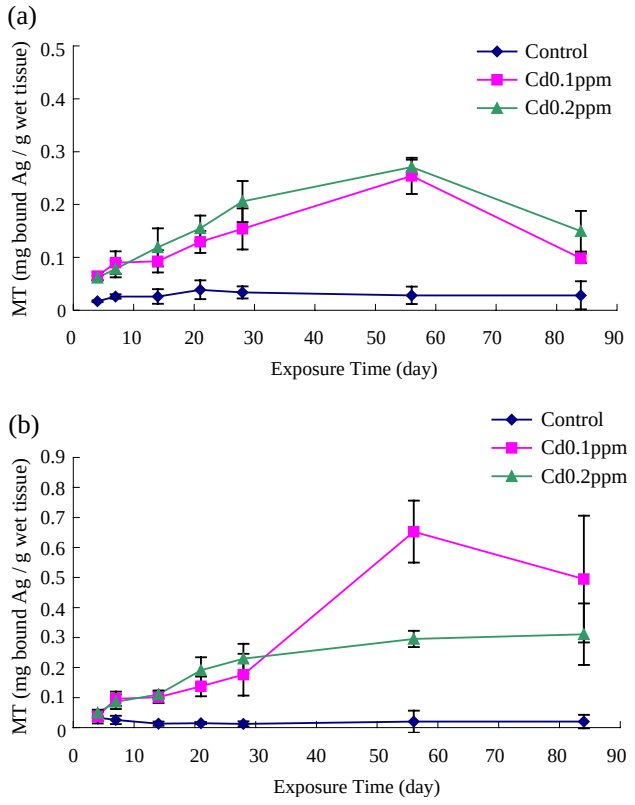

Fig. 3. Metallothionein concentrations measured in (a) the hepatopancreas and (b) gills of *L. vannamei* using the silver saturation method after animals were exposed to cadmium for 84 days.

trations were measured using a polarized Zeeman atomic absorption spectrophotometer (Hitachi model Z-8100, Tokyo, Japan) (EPA/ROC, 1994).

There were at least four important conditions to ensure the stability of MT extracted from tissue samples during storage, including heat-treatment of homogenates, storage of supernatants at −70 °C, the addition of a thio-protecting agent to the supernatants, and maintenance of sample dilutions to no greater than 1:5 (Coyle et al., 2001). Since our samples were handled fresh, we needed to use DTT as the sulfydryl-protecting agent to prevent the MT from oxidation, as well as PMSF to prevent protease activity, regardless of the storage temperature. Additionally, since MT is heat-resistant, heat-treatment removed all other proteins, which are denatured due to the high temperatures after centrifugation, except for MT.

2.4. Statistical analyses

Statistical analysis was performed with one-way analysis of variance (ANOVA) to determine the treatment and time effects on MT induction and metal accumulation after exposure to heavy metals. Duncan's multiple-range test was used to evaluate the mean difference among individual groups at a 0.05 significance level. Results are reported as the mean ± the standard deviation (SD).

3. Results

3.1. MT separation patterns

The Sephadex G-75 (superfine) elution profiles of heat-stable proteins with metals derived from hepatopancreatic tissue of control, zinc-, and cadmium-treated shrimp showed interesting patterns (Fig. 1). In the profile of control shrimp (Fig. 1a), there were two clear protein peaks, peaks I and III, as well as one indistinct protein peak, peak II. The first protein peak corresponded to the Zn peak and a slight Cu peak, and the third peak showed a correspondence to the Zn peak, while the second peak only corresponded to a slight Cu peak. After exposure to cadmium, the peak pattern became clearer and more distinguishable (Fig. 1b). The first protein peak corresponded not only to the Zn and a slight Cu peak but also to the Cd peak. The second peak became more obvious and corresponded to the Cu peak as well as the Cd peak. However, the third protein peak corresponded only to the Zn peak regardless of whether the shrimp had been exposed to Cd. After exposure to zinc, the elution profile was similar to that of control shrimp, i.e., there were three protein peaks: the first one corresponded to the Zn and a slight Cu peak, the second one corresponded to a slight Cu peak, with the third one showing correspondence to the Zn peak (Fig. 1c). In addition, the patterns of elution profiles on the Sephadex G-50 column showed similar behavior to

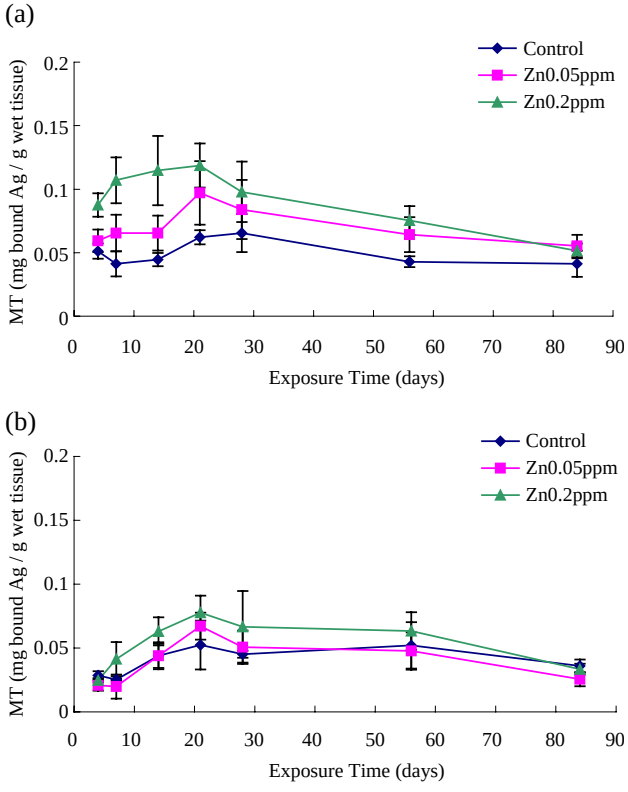

Fig. 4. Metallothionein concentrations measured in (a) the hepatopancreas and (b) gills of *L. vannamei* using the silver saturation method after animals were exposed to zinc for 84 days.

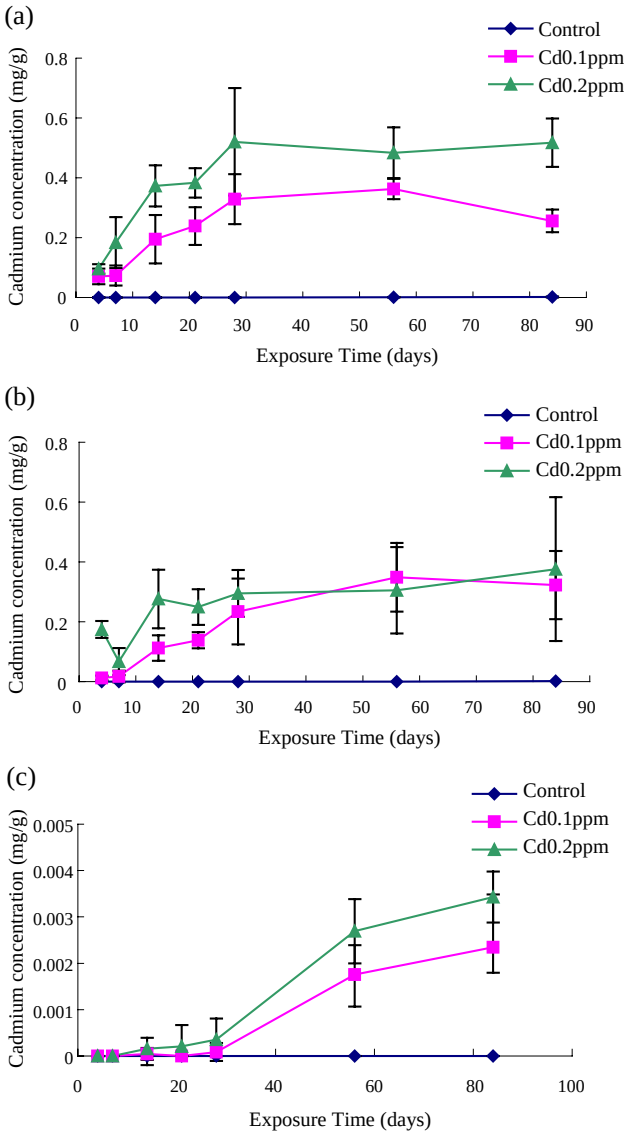

Fig. 5. Cadmium concentration measured in (a) the hepatopancreas, (b) gills, and (c) muscle tissues of *L. vannamei* after animals were exposed to cadmium for 84 days.

those on the Sephadex G-75 (superfine) column (Fig. 2), although the last two protein peaks were less obvious, due to the dilution effect of the larger total elution volume. On the other hand, the results of molecular weight determination showed that these three metal-associated protein peaks had native molecular weights of 37,700, 16,500, and 1600, respectively (R^2=0.9948).

3.2. Animal activities during the period of long-term exposure

Throughout the experimental period of long-term exposure, uncoordinated, moribund, and dead shrimp as defined by Cardeilhac et al. (1979) in their studied animal, the sheephead *Archosargus probatocephalus*, became apparent and were observed after 56 days treated with 0.2 mg Cd/l and 84 days treated with 0.1 mg Cd/l, while similar circumstances were not observed in zinc-treated or control shrimp.

3.3. MT concentrations within the hepatopancreas and gills

The effect of cadmium on MT induction in the hepatopancreas and gills is shown in Fig. 3. In the treatment of shrimp exposed to 0.1 mg Cd/l, there was a steady time-dependent increase in the amount of MT induced within the hepatopancreas until day 56 when the highest value of 0.254±0.034 mg bound Ag/g wet tissue was observed (Fig. 3a). However, after exposure for 84 days, the amount of MT induced had fallen to 0.149±0.009 mg bound Ag/g wet tissue. In treatment with 0.2 mg Cd/l, the amount of induced MT within the hepatopancreas increased more sharply until day 28, while the value at day 56 was 0.271±0.014 mg

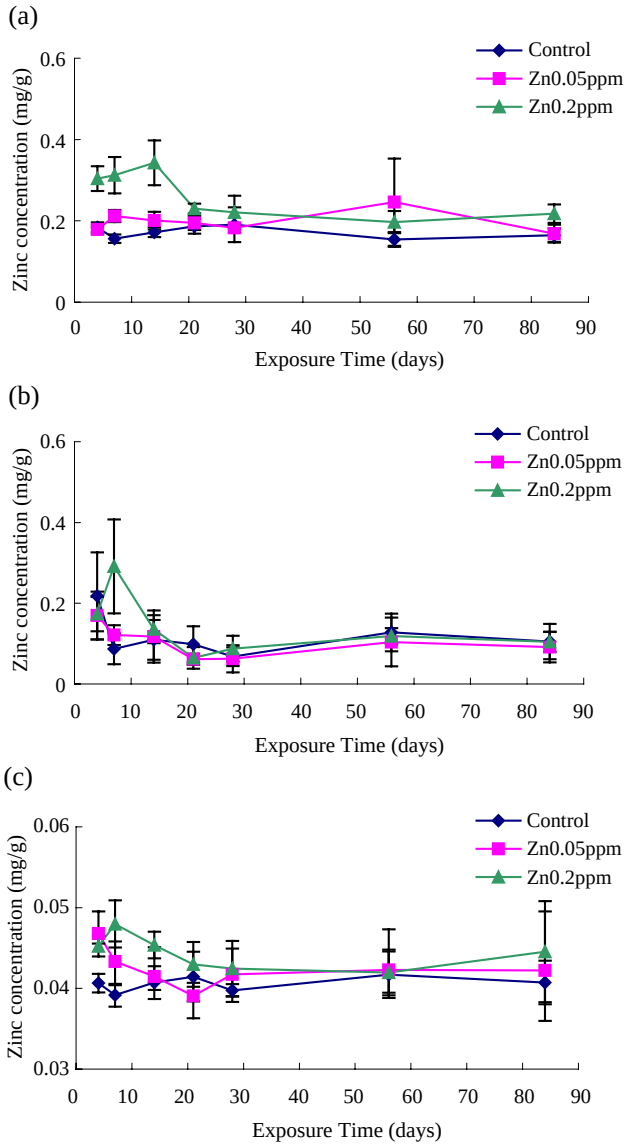

Fig. 6. Zinc concentration measured in (a) the hepatopancreas, (b) gills, and (c) muscle tissues of *L. vannamei* after animals were exposed to zinc for 84 days.

bound Ag/g wet tissue, and a tendency to increase was not seen, with no significant difference with that induced by 0.1 mg Cd/l at 0.254±0.034 mg bound Ag/g wet tissue ($p>0.05$), while the value on treatment day 84 had fallen to 0.098±0.009 mg bound Ag/g wet tissue.

A similar picture emerged from the gills with the same treatment (Fig. 3b). Until day 28, there were time-and dose-dependent increases in the amount of MT induced in both 0.1 and 0.2 mg Cd/l treatments. However, after the shrimp had been treated for 56 days, the amount of MT induced by 0.2 mg Cd/l exposure (0.296±0.027 mg bound Ag/g wet tissue) was significantly lower than that induced by 0.1 mg Cd/l (0.652±0.103 mg bound Ag/g wet tissue) ($p<0.05$). The value with 0.1 mg Cd/l treatment after 84 days of exposure gradually declined to 0.495±0.211 mg bound Ag/g wet tissue, while that with 0.2 mg Cd/l treatment remained steady at 0.311±0.102 mg bound Ag/g wet tissue.

It was very interesting that the dose- and time-dependent elevations we observed in the results of Cd treatments were not apparent in the results of MT induction within the hepatopancreas and gills of shrimps exposed to zinc (Fig. 4). Within the hepatopancreas, the amount of MT induced reached at the highest level of 0.119±0.017 mg bound Ag/g wet tissue after exposure to 0.2 mg Zn/l for 21 days and then dropped to 0.051±0.06 mg bound Ag/g wet tissue; this did not significantly differ from that of control shrimp exposed for 84 days ($p>0.05$) (Fig. 4a). Also, a similar behavior was observed when shrimp were exposed to 0.05 mg Zn/l. Likewise, the amount of MT induced in the gills of 0.2 mg Zn/l-treated shrimp reached a significantly higher level than that of control shrimp as well as 0.05 mg Zn/l-treated shrimp at the exposure times of 4, 7, and 14 days ($p<0.05$) (Fig. 2b).

Throughout the experimental period, the amounts of MT appearing in control animals were steady at 0.039±0.014 mg bound Ag/g wet tissue in the hepatopancreas and 0.031±0.017 mg bound Ag/g wet tissue in gills.

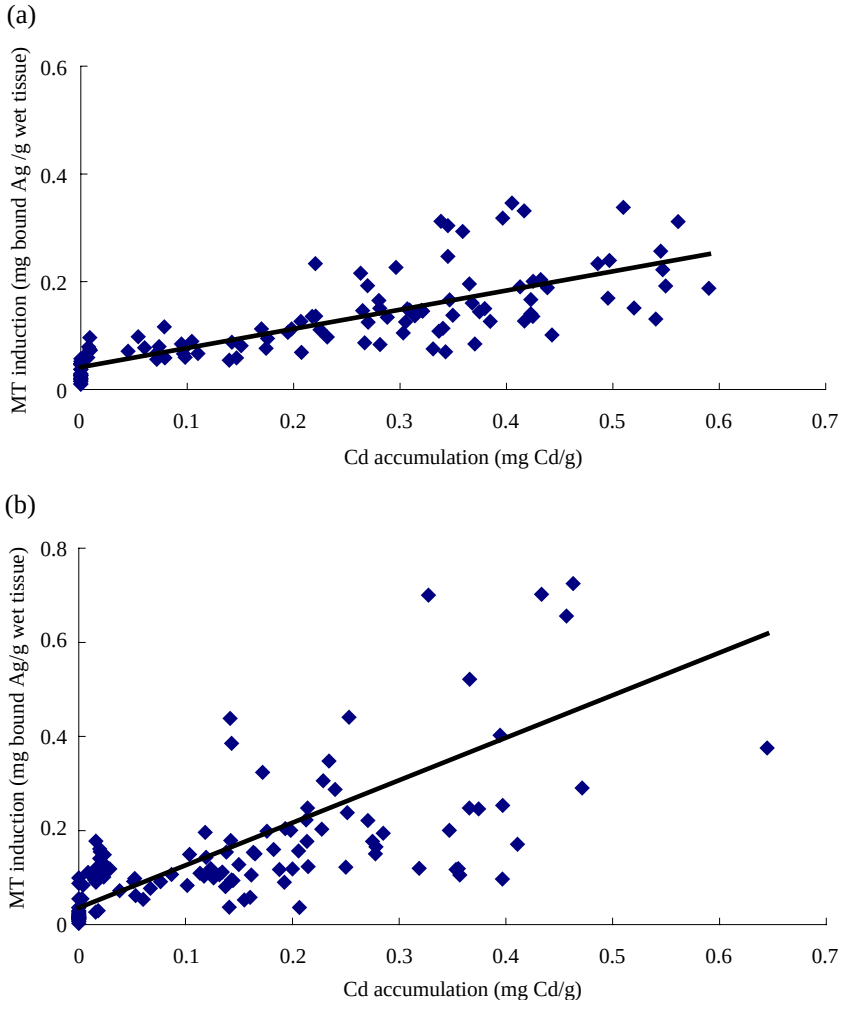

Fig. 7. Correlation between MT concentration and cadmium concentration in (a) the hepatopancreas of *Litopenaeus vannamei*, with a linear regression equation of $Y=0.356*X+0.041$ ($R^2=0.618$; $P<0.0001$), and (b) gills, with a linear regression equation of $Y=0.904*X+0.036$ ($R^2=0.416$; $P<0.0001$) throughout the experimental period when animals were exposed to cadmium.

3.4. Metal concentrations within the hepatopancreas, gills, and muscle tissues

After exposure to Cd, the hepatopancreas, gills, and muscles each accumulated Cd with particular patterns (Fig. 5). After exposure to Cd, the hepatopancreas accumulated Cd gradually until 28 days with values of 0.329±0.083 mg Cd/g with 0.1 mg Cd/l treatment and 0.520±0.179 mg Cd/g with 0.2 mg Cd/l treatment, both of which showed significant differences with that of control shrimp ($p<0.05$) (Fig. 5a). However, after exposure for 28 days, the amount of Cd accumulating within the hepatopancreas remained at a steady level both in the 0.1 and 0.2 mg Cd/l treatments. The results of accumulation in gills after white shrimp were exposed to Cd showed irregular behavior (Fig. 5b). Although there was a tendency for the concentration to increase, the amount of Cd which accumulated within the gills fluctuated throughout the experimental period with wide standard deviations, especially for shrimp treated with 0.2 Cd mg/l. The accumulation of Cd in white shrimp muscles was light, with regard not only to the accumulation rate but also to the quantity of Cd accumulated (Fig. 5c). The amount of accumulated Cd steadily increased until the end of the experiment with the highest value of 0.0023±0.0011 mg Cd/g in the 0.1 mg Cd/l treatment and 0.0034±0.0005 mg Cd / g in the 0.2 mg Cd/l treatment.

Patterns of Zn accumulation in the hepatopancreas and gills were similar to those of MT induction after shrimp were exposed to Zn. Significantly higher levels of Zn accumulating within the hepatopancreas were observed when shrimp were exposed to 0.2 mg Zn/l for 4, 7, and 14 days, as well as when shrimp were exposed to 0.05 mg Zn/l for 7 days ($p<0.05$) (Fig. 6a). However, the amount of Zn appearing within the hepatopancreas then declined to levels that showed no significant difference compared with those of the control shrimp. A similar picture was also observed for the results of Zn accumulation within gills from treated shrimp, with a significantly high level of Zn accumulating when shrimp were exposed to 0.2 mg Zn/l for 7 days ($p<0.05$) (Fig. 6b). Otherwise, the quantities of Zn accumulating within muscles of Zn-treated shrimp showed almost no significant difference with those of control shrimp ($p>0.05$) (Fig. 6c).

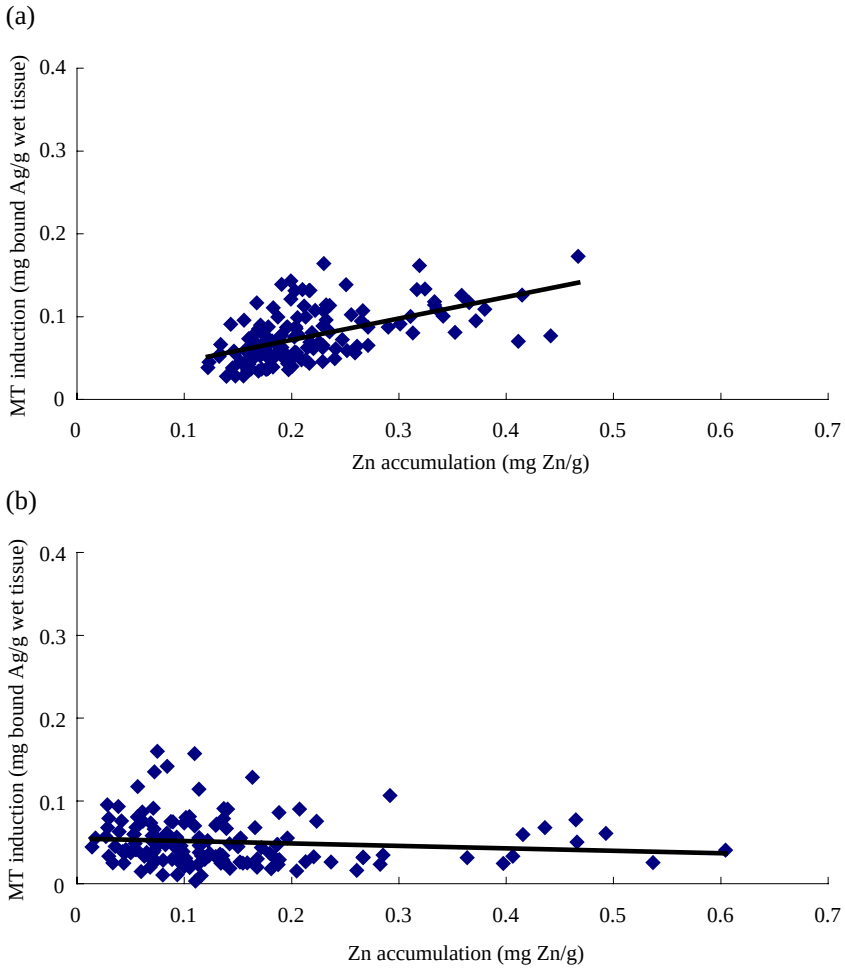

Fig. 8. Correlation between MT concentration and zinc concentration in (a) the hepatopancreas of *L. vannamei*, with a linear regression equation of $Y=0.260*X+0.020$ ($R^2=0.303$; $P<0.0001$), and (b) gills, with a linear regression equation of $Y=-0.030*X+0.055$ ($R^2=0.012$; $P=0.191$) throughout the experimental period when animals were exposed to zinc.

3.5. Correlation between MT concentrations and metal concentrations during the period of long-term exposure to Cd and Zn

Additionally, a positive correlation was shown between the amount of MT induced and the amount of Cd accumulated. Regression equations were determined for the amount of MT induced, and were 0.356*(the amount of Cd accumulated)+0.041 (R^2=0.617; P<0.0001) in the hepatopancreas, and 0.904*(the amount of Cd accumulated)+0.036 (R^2=0.416; P<0.0001) in gills (Fig. 7). However, the amount of MT induced showed a relatively lower correlation with the amount of Zn accumulated. The following regression equations were determined for the amount of MT induced, and were 0.260*(the amount of Zn accumulated)+0.020 (R^2=0.303; P<0.0001) in the hepatopancreas, and −0.030*(the amount of Zn accumulated) +0.055 (R^2=0.012; P=0.191) in gills (Fig. 8).

4. Discussion

According to the elution profiles of gel filtration chromatography using the Sephadex G-75 (superfine) column, which can separate molecules of different molecular weights ranging from Mr. 3000–70,000, and the Sephadex G-50 column, which has a separation range of from Mr. 1500–30,000, it is clear that there were three size-separated proteins with similar properties of heat stability and metal association ability, both unique properties of MTs. Among these three metal-associated proteins, the first and the second protein peaks should be the MTs or MT-like proteins of *L. vannamei*, which are associated with Zn, Cu, and Cd, while the third protein peak is a Zn peak that is not associated with any metal ions other than Zn. The results of our study are similar to the account given by Wong and Rainbow (1986) working on the shore crab, *Carcinus maenas*. Multiple forms of MTs have been identified in most aquatic animals in which MTs have been characterized. Some animals have two MTs, such as rainbow trout and crab, while others have three, such as the lobster (Roesijadi, 1992). Each specific form of MT of different species may have unique functions, some related to copper donation reactions, some related to metal detoxification, others related to metal requirements for growth and cellular differentiation, as mentioned by Roesijadi (1992) in his review article.

We observed no significant change or increase in the quantity of the MTs or MT-like proteins after the shrimp were exposed to metals when we tried to identify MTs or MT-like proteins (Figs. 1 and 2). We assumed that the metal concentrations and exposure times before sacrificing the animals were still too limited to trigger the de novo mass induction of MTs or MT-like proteins in the hepatopancreas of *L. vannamei*. However, as was mentioned by Smet et al. (2001), the phenomenon of cadmium occupying the unbound binding sites or even competing with zinc or other elements for available binding sites on the MT or MT-like proteins after exposure to cadmium was apparent in our results. This was followed by increased concentrations of cadmium and even decreased concentrations of zinc within MT or MT-like protein fractions in the elution profiles (Figs. 1b and 2b). Furthermore, we observed that when animals were exposed to zinc, the zinc continued to bind to the available binding sites on the MT or MT-like proteins and the concentration of zinc within MT or MT-like protein fractions increased (Figs. 1c and 2c).

Many methods have been developed to estimate the quantities of MTs within the whole body or organs of target organisms. Although the enzyme-linked immunosorbent assay (ELISA) method has the advantage of showing higher specificity to target specimens, the largest problem lies in the cost and availability of species-specific antibodies of MTs. Likewise, safety problems often limit the usage of the radioimmunoassay (RIA) method, although this assay always shows results with high sensitivity. Among methods commonly used to estimate the quantities of MTs, the metal saturation method has the advantages of being more conveniently and easily manipulated, and is inexpensive, useful, and safe. Although the metal saturation method, which uses the quantity of metals bound with MTs after extraction to indirectly reflect the quantities of MTs, sometimes tends to overestimate MT concentrations in tissue specimens, yet the statistical comparison of this indirect method with a direct one revealed that they yielded similar results and trends (Pederson et al., 1997).

We observed that MTs existed within organs of normal *L. vannamei* we examined with a basal level of about 0.03 ~ 0.04 mg bound Ag/g wet tissue. MTs, which are cytosolic proteins, are involved in the normal physiology of decapods. For example, MTs can transfer copper to the respiratory pigment, hemocyanin, as well as regulate copper levels during the molting cycle (Bainy, 2000). Also, MTs bind to nonessential metals, such as Cd and Hg, which represents a sequestration function for protection against metal toxicity (Roesijadi, 1992).

After a short-term exposure to Cd for about 1 month, our results from *L. vannamei* were in complete agreement with those of many other investigations that the induction of MTs shows dose- and time-dependence, such as the work done by Martinez et al. (1993) on the crayfish, *Procambarus clarkii.* The presence of heavy metals activates the transcription of MT genes via the binding of metal-binding regulatory factors (MRFs) to the metal-responsive elements (MREs) (Roesijadi, 1992). In fish, the level of MT-mRNA increases following administration of heavy metals (Bonham and Gedamu, 1984; Olsson et al., 1989; Hogstrand and Haux, 1991). After exposure to nonessential heavy metals like Cd and Hg, the induction of MTs increases the binding of heavy metals to the protein, which serves a sequestration function to decrease the toxicity of Cd and Hg; it has been suggested that toxic effects of Cd and Hg only occur when

the binding capacity of the MT is exceeded and these metals appear in the high molecular weight proteins of the cytosol (Hogstrand and Haux, 1991; Klaassen, 2001).

However, circumstances differed when shrimp were exposed to Cd for longer than 1 month (Fig. 3). The increase in the level of induced MTs was retarded with a second month of exposure time, and this phenomenon was very obvious especially in shrimp exposed to the higher doses. Furthermore, the level of induced MTs decreased after the shrimp were exposed to Cd for 3 months. We considered that it was most likely the toxicity of Cd to shrimp which caused the decrease in the levels of MTs. It is well known that heavy metals cause impacts on normal structures and functions of some organs in aquatic organisms, leading to changes in normal metabolism and physiology. For example, some heavy metals can cause structural changes in the gills of many aquatic organisms and influence their oxygen uptake ability (Matthiessen and Brafield, 1973; Soegianto et al., 1999a,b). Crustaceans under hypoxia will change their energy substrates from lipids to proteins (Rosas et al., 1999; Chinni et al., 2000). Also, heavy metals will cause morphological and functional alterations of the intestines of aquatic organisms, which may lead to some effects on absorption of nutrients and ions (Crespo et al., 1986). Additionally, Cd causes cytotoxicity in the hepatocytes of rats (Koizumi et al., 1994), and cytotoxicity should occur in cells of the hepatopancreas as well as the gills of *L. vannamei*. There is a close relationship between the total amount of Cd and the reduction in protein synthesis of cells (Din and Frazier, 1985; Hogstrand and Haux, 1991). Our results showed that the higher dose the animals were exposed to, the more-serious effects that were caused. The toxicity of Cd caused not only decreases in the level of MTs but also weakness and even death of *L. vannamei* after long-term exposure.

Most aquatic animals absorb heavy metals via the gills and intestines, and then transfer them to the blood and other parts of the body. It has been demonstrated that Cd slowly accumulates in fish, and that the major targets for Cd distribution are the kidney and liver (Hogstrand and Haux, 1991). At the subcellular level, the majority of intracellular Cd exists in the cytosol and is associated with MT, according to the results of gel filtration chromatography of the cytosol. In *L. vannamei*, the major organ in which Cd accumulates is the hepatopancreas according to the results from examination of shrimp caught both from the wild and from farms located on the northwestern coast of Mexico (Paez-Osuna and Tron-Mayen, 1996), and which are similar to those of many crab species (Turoczy et al., 2001). After exposure to Cd, the hepatopancreas of *L. vannamei* accumulated Cd with dose- and time-dependence during the first month (Fig. 5a). However, the Cd concentration within the hepatopancreas stabilized when the exposure time was longer than 1 month, due to the retardation of MT induction and the saturation of Cd-binding sites of the MTs. Otherwise, Cd existed within gills with a quite-complicated and fluctuating pattern (Fig. 5b). Gills are the major entry site of metals and act as a transient store for accumulated metals (Soegianto et al., 1999a). Additionally, it is known that the gills of aquatic animals have the ability to excrete invading heavy metals (Nakatani, 1966). The final quantity of Cd within gills we examined depended on the result of interactions between absorption, excretion, and transfer of Cd to other tissues. Compared to the hepatopancreas and gills, Cd distributed within muscle tissues had lower concentrations, which were about 1% lower than that of gills, although it did show dose- and time-dependence (Fig. 5c).

Unlike Cd, Zn showed a limited ability to induce de novo MT synthesis, and the maximum concentration of induced MT within tissues was lower than the 0.15 mg bound Ag/g wet tissue in the hepatopancreas and the 0.10 mg bound Ag/g wet tissue in gills (Fig. 4). MT induction of Zn-exposed shrimp was significantly evident in the first few weeks, after which no significant difference was shown with that of control shrimp. Likewise, the time-course of Zn accumulation within tissues of Zn-exposed shrimp showed similar behavior to that of MT induction (Fig. 6a,b). Being a ubiquitous and nutritionally essential metal for animals, Zn also has the ability to induce the synthesis of MT, which is a factor in regulating the metabolism of Zn, including absorption and storage (Klaassen, 2001). On the other hand, Vijayram and Geraldine (1996) demonstrated that the freshwater prawn, *Macrobrachium malcolmsonii,* appeared to possess a physiological mechanism that regulated tissue Zn concentrations within certain limits until Zn exposure dosage or time exceeded the threshold and the regulation mechanism collapses. Marine prawns are also believed to possess the ability to regulate tissue Zn concentrations for normal physiology (White and Rainbow, 1985; Vijayram and Geraldine, 1996). Hence, we consider that the time–course pattern of Zn existence appearing in our results from exposed *L. vannamei* was caused by their regulation mechanism of Zn distribution, in which MTs should be involved. It was reported that MT-bound Cd has an extremely long half-life and the degradation of Cd–MT induces the synthesis of additional new MT proteins, while MT-bound Zn shows higher rates of turnover than Cd (Langston et al., 1998; Nassiri et al., 2000). In other words, Zn stays MT-bound in the cytosol for less time than Cd, and then appears in lysosomes degrading MT, and does not induce additional de novo synthesis of MT. This should be one of reasons why Zn showed limited ability to induce additional de novo MT synthesis, and the concentration of accumulated Zn in tissues of Zn-exposed shrimp decreased to the level showing no significant difference with that of control shrimp. Finally, it seemed that the Zn concentration we used to treat *L. vannamei* was below the threshold, and they still had the ability to maintain their optimal Zn distribution within tissues or organs, such that the largest percentage of body Zn existed in the muscle; additionally, the highest Zn concentration actually appeared in the hepatopancreas (Paez-Osuna and Tron-Mayen, 1996).

According to the suggestions given by Pederson et al. (1997), there are three important issues which need to be considered before biomarkers are used in routine environmental management and monitoring, including (a) Do biomarker concentrations in tissues reflect the extent of chronic environmental contamination?; (b) Do biomarker concentrations in specific tissues reflect contaminant concentrations in those tissues?; and (c) Are the responses of different biomarkers consistent with one another? To use MTs as biomarkers to reflect the existence of heavy metal contamination, the relationship between MT induction and metal accumulation should be considered and examined. Actually, using the polarographic method, Moksnes et al. (1995) demonstrated the phenomenon of MT induction in *L. vannamei* exposed to Cd with a dose–response experiment in which shrimp were exposed to six different concentrations of waterborne $CdCl_2$ from 0 to 2.00 mg Cd/l for 6 days, and a time–course experiment in which shrimp were exposed to 1.50 mg Cd/l for 21 days and sampled at 9, 14 and 21 days. Following their results, the potential for MT induction to be used for monitoring and evaluation the heavy metal pollution in shrimp farms was reasonably considered (Bainy, 2000). However, metal concentrations within tissues of *L. vannamei* were not examined in former experiments. Additionally, an exposure time of 21 days is too short for assessment of the suitability of using MT induction in field applications, because it generally takes about 3 ~ 4 months to rear prawns from the postlarval stage to market size on shrimp farms. Indeed, our results confirmed that MT induction showed a good linear dose- and time-dependence in the first month after *L. vannamei* exposure to Cd, as well as did Cd accumulation within the hepatopancreas. However, the relative amounts of intercellular heavy metals associated with MTs are strongly dependent on the degree and duration of the exposure as well as the tissue examined (Hogstrand and Haux, 1991). The phenomenon of MT induction and Cd accumulation being retarded or remaining stationary after *L. vannamei* were exposed to Cd for more than 1 month may cause underestimation of the effects or exposure times. Also, Cu, Zn, Cd, and Hg induce and interact with MT in all species investigated, but the metals bind to MTs to varying degrees (Hogstrand and Haux, 1991). At the least, Zn showed a different MT-induction behavior compared with Cd according to our results. These are all issues which need to be discreetly considered when using MT induction as a biomarker to reflect metal contamination in environmental applications. Roesijadi (1992) reviewed the role that MT plays in metal regulation in aquatic animals and discussed arguments against application of MT analysis in environmental studies due to a lack of the information about the exact relationship between MT induction and heavy metal exposure. Also, for these purposes to be achieved requires more information in order to understand MT's functions and the limits of MT induction, as well as the relationships between levels of induction and the behavior of protected target systems. Additionally, it is better to measure the rate of MT and MT mRNA synthesis and metal-binding which may be more informative than to measure concentrations of MT, MT mRNA, or metal-binding alone; future work should be devoted to determining these aspects.

5. Conclusions

Although it is still not completely clear what the exact functions and structures of each form of MTs are, we were still able to identify at least two MT-like proteins of different sizes in *L. vannamei*. After determining the existence of MTs or MT-like proteins in *L. vannamei*, we also demonstrated that Cd and Zn show different MT induction abilities in different organs after long-term exposure. In addition, it was also observed that different organs of *L. vannamei* accumulate Cd and Zn in different patterns. However, more needs to be considered before MTs can be used as biomonitors to reflect the existence of heavy metals in prawn culture ponds.

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