Vol. 23(4):337-345 | Ocean and Polar Research | December 2001

Article

Preliminary Evidence for a Metallothionein-like Cd-binding Protein in the Kidney of the Antarctic Clam *Laternula elliptica*

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Abstract : A Cd-binding protein was identified in the renal cytosol of the Antarctic clam *Laternula elliptica* which naturally contains high concentrations of Cd. The Cd-binding protein showed similar characteristics of metallothionein (MT) in molecular weight (about 10-12 kDa) and low spectral absorbance at 280 nm with relatively high absorbance at 254 nm. Results of immuno-histochemical staining suggested that the MT-like Cd-binding protein was mainly located in the epithelial cells of the kidney. The MT-like protein was a major ligand of cytosolic Cd as shown in the elution profiles of chromatography and may play an important role in Cd sequestration and accumulation in *L. elliptica* kidney. A considerable amount of Cd was also found to be associated with particulate fraction, indicating the sequestration to particulate fraction is as important as binding to the cytosolic MT-like protein in Cd accumulation in the kidney.

Key words : *Laternula elliptica*, cadmium, metallothionein-like protein, kidney, Antarctic clam.

1. Introduction

Cadmium is one of the most toxic heavy metals in aquatic environments (Wittmann 1981; Langston 1990; Luoma and Carter 1991; Chelomin *et al.* 1995). The Antarctic Ocean, though the most pristine environment on earth, contains several times higher concentration of Cd compared to other clean open ocean, not because of anthropogenic impacts but mainly due to a natural process, the upwelling of the Cd-enriched deep water (Honda *et al.* 1987; Bargagli *et al.* 1996; Nigro *et al.* 1997). Reflecting the high Cd concentration in seawater, organisms in the Antarctic marine ecosystem also contain high levels of Cd in their tissues. Especially in mollusc tissues, Cd is accumulated up to 10^5 times more than in the seawater (Bargagli *et al.* 1996; Nigro *et al.* 1997). In spite of such high body burdens of Cd, the organisms have persisted through the geologic times in one of the most extreme environments of the world, the Antarctic Ocean (Berkman 1997). Such a finding suggests that these organisms must have developed effective cadmium-detoxifying mechanisms.

Production of metal binding ligands in cells, such as metallothionein (MT), is one of the most commonly adopted mechanisms for metal detoxification in organisms in temperate marine environments (Mason and Jenkins 1995; Langston *et al.* 1998). Metallothioneins are a class of sulfhydryl-rich, low molecular weight proteins that show strong affinity for class B metals such as Cd, Ag, Cu, and Zn (Roesijadi 1992; Stillman 1995; Langston *et*

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al. 1998). By rapidly sequestering non-essential or excessive essential metals, MT removes the free metal ions from the cells (Mason and Jenkins 1995; Langston *et al.* 1998). Presence of MT or MT-like protein (MTLP) in metal accumulated tissues of various mollusc species has been extensively reported, since the first invertebrate MTs of the oyster *Crassostrea virginica* and the mussel *Mytilus edulis* were described in the mid 1970's (Roesijadi 1992; Viarengo and Nott 1993; Mason and Jenkins 1995; Langston *et al.* 1998, for reviews). Inducibility of MT or MTLP after exposures to excess metals imply that these proteins perform a crucial function in metal detoxification in temperate marine molluscs (Roesijadi 1992; Bebianno and Langston 1995; Duquesne *et al.* 1995; Leung and Furness 1999, 2001).

Studies on MT or similar metal-binding proteins in Antarctic organisms have set about only recently (Carginale *et al.* 1998; Bargelloni *et al.* 1999; Scudiero *et al.* 1997, 2000; Santovito *et al.* 2000), and the importance of these proteins in homeostasis and detoxification of Cd in Antarctic molluscs is still in question. Currently data on metal-binding proteins from Antarctic mollusc are available for one species only, the Antarctic scallop *Adamussium colbecki*. An MT-like Cd-binding protein was detected in the digestive gland of *A. colbecki*, where high concentration of Cd was found (Viarengo *et al.* 1993, 1997; Ponzano *et al.* 2001). The concentration of the MTLP increased when the animals were exposed to high concentration of Cd, suggesting the involvement of this protein in the cellular Cd detoxification in this species (Ponzano *et al.* 2001).

The Antarctic clam *Laternula elliptica* is one of the most abundant macrobenthic fauna in shallow Antarctic waters (Ahn 1994a,b). By virtue of its wide distribution, large body size, and high population density in addition to strong tendency of metal accumulation, this bivalve species is recognized as a sentinel species for monitoring environmental contamination in Antarctic waters along with the Antarctic scallop *A. colbecki* (SCAR/COMNAP 1996; Ahn *et al.* 1996, in press; Nigro *et al.* 1997). Extremely high accumulation of Cd was found in the kidney of *L. elliptica* and Cd binding to MT in the kidney cells was suggested as the cause for it in a previous study (Ahn *et al.* 1996). However, the presence of MT or MT-like protein in this species has never been confirmed. As a first step for understanding Cd detoxification processes in the Antarctic clam *L. elliptica*, the presence of metallothionein-like Cd-binding protein in the kidney was examined in this study. An MT-like protein was isolated biochemically and its location in the kidney cells was identified using an immuno-histochemical technique.

2. Materials and methods

Sample preparation

Laternula elliptica were collected at depths of 25-30 m near King Sejong Station located in Maxwell Bay, Antarctica in November 1998 and July 1999. For biochemical analysis of MT and metal analysis the clams were kept frozen at ⇦70 °C until they were dissected into kidney, gill, digestive gland, gonad, and muscle parts on ice. Wet weight of each tissue part was measured and immediately frozen at ⇦70 °C. Two to four kidneys from the specimens of 65-85 mm in shell length were pooled so that the wet weight of each sample should be about 3-5 g. For detecting MT in the kidney immunohistochemically, several additional clams were dissected and their kidney was fixed with 2 % glutaraldehyde buffered with 1 M phosphate (pH 7.2) and kept at 4 °C until further analysis.

Subcellular fractionation

The kidney tissue samples were homogenized in 2 or 3 volumes of 150 mM NaCl, 20 mM Tris-HCl (pH 8.6) containing 0.5 mM PMSF (Phenylmethylsulphonyl fluoride) and 0.01 % ⊖mercaptoethanol in ice. The buffer was bubbled with N_2 gas to strip out any O_2 in it before the homogenization. The homogenate of known volume was then centrifuged at 100,000 g for 2 hour at 4 °C and the volumes of the supernatant (cytosolic fraction) and the pellet (particulate fraction) were measured.

Determination of Cd concentration

Cadmium concentrations in the total homogenate, cytosolic fraction, and particulate fraction were determined with a graphite furnace atomic absorbance spectrophotometer (HGA800, Perkin Elmer). Aliquots of the samples were digested with 65 % HNO_3 (Merck) using a microwave system (MDS-2000, CEM), then filtered and diluted before the measurement with AAS. The accuracy of the analytical method was tested using the oyster standard material 1566a of NIST, USA. The percentage of recovery of SRM was 93-94 %.

Analysis of Cd-binding metallothionein

Two milliliters of cytosolic fraction were applied to a 2.7×37 cm Sephacryl S-100 (LKB Pharmacia) column which was calibrated with Vitamin B_{12} (1.35 kDa), rabbit

liver metallothionein (6.5 kDa), and α-chymotrypsin (25 kDa) and eluted with 20 mM Tris-HCl (pH 8.6) containing 0.01 % β-mercaptoethanol at a flow rate of 0.79 ml/min at 4 ℃. Three ml fractions were collected and the absorbance at 254 nm and 280 nm were measured. The cadmium concentration of each fraction was determined directly (no acid digestion) using a graphite furnace AAS. Fractions with the highest Cd concentrations were pooled and applied to a 1.5×6 cm DEAE Sepharose ion exchange column (LKB Pharmacia). Proteins were eluted with linear concentration gradient of Tris-HCl (pH 8.2, containing 20 mM β-mercaptoethanol) from 20 mM to 800 mM at a flow rate of 2.3 ml/min. Two ml fractions were collected and the Cd concentration of each fraction was measured.

Total homogenate, cytosolic fraction, and the Cd-containing fractions collected after the gel filtration chromatography and ion exchange chromatography were analyzed by sodium dodecyl surflate (SDS)-polyacrylamide gel electrophoresis (PAGE) based on the method by Chung & Bryant (1996). Each sample was mixed with loading buffer and boiled at 95 ℃ for 5 min, then loaded to 16 % Schägger gel. The electrophoresis was conducted at 100V using 0.2 M Tris-HCl (pH 8.9) for the bottom running buffer and 0.1 M Tris, 0.5 M Tricine containing 0.5 % SDS (pH 8.2) for the top buffer in a Mini-PROTEIN® electrophoresis kit (BIO-RAD), and then the gel was silver stained. Total protein concentrations of total homogenate, cytosolic fraction, Cd-containing fractions obtained from chromatography were assessed based on Bradford method using bovine serum albumin as a standard.

Immunohistochemical detection of metallothionein

The kidney tissue samples fixed with 2 % glutaraldehyde were washed with 1 M phosphate buffer (pH 7.4) and dehydrated in a graded series of ethanol from 50 to 100 % followed by embedment in paraffin. The embedded samples were stored at ⇦20 ℃ until they were sectioned to 6 μm with a rotary microtome and mounted on the glass slides. The following processes were conducted using LSAB kit from DAKO. The tissue sections on the slides were de-paraffined in xylene, rehydrated in a series of ethanol, and rinsed in phosphate buffer. Endogenous peroxide activity was blocked with 3 % H_2O_2 in PBS for 5 min. The sections were incubated in a blocking solution for 5 minutes to prevent any nonspecific bindings before being incubated in a monoclonal mouse metallothionein antibody (DAKO) for 10 min and treated with a secondary antibody biotinylated anti-mouse immunoglobulins (DAKO) for 10 min at room temperature. The samples, which were incubated in streptavidin peroxidase solution and substrate-chromogen solution for 10 minutes respectively, were counter stained with hematoxylin when necessary and then were mounted with DAKO glycerge mounting medium. The prepared specimens were observed under Nikon Optiphot-II light microscope.

Table 1. Cadmuim concentrations in subcellular fractions of *Laternula elliptica* kidney (μg g^{-1} tissue dry wt) collected in an austral summer (November 1998) and in a winter (July 1999). Values are ± mean standard deviations (n=5). The symbol * indicates significant difference between summer and winter at p=0.05 (Non-parametric Mann-Whitney U-test).

Subcellular fraction	Summer	Winter
Total homogenate	113.09 ± 22.20	130.36 ± 19.07
Cytosolic fraction*	42.18 ± 17.70	22.28 ± 1.04
Particulate fraction*	70.05 ± 13.05	105.50 ± 21.14

3. Results

Cd concentrations in the kidney

Cadmium concentrations in the whole kidney homogenates, cytosolic fractions and insoluble particulate fractions are summarized in Table 1. The concentration of Cd in the cytosolic fraction was higher in the summer than in the winter (Mann-Whitney U-test, p=0.051). In particulate fraction, the reverse situation was found showing higher concentrations in the winter (Mann-Whitney U-test, p= 0.051). Overall, the Cd concentration in the total homogenate showed no seasonal difference (Mann-Whitney U-test, p>0.2) and the numbers ranged between from 90 to 150 μg g^{-1} tissue dry weight.

There was a tendency that more Cd was distributed in the particulate fraction than in the cytosolic fraction both in the summer and winter samples, but the difference was not statistically significant probably due to the small sample size (Mann-Whitney U-test, p=0.06 for the summer samples and p=0.08 for the winter samples).

Characterization of metal-binding proteins in the cytosol

A representative Sephacryl S-100 chromatogram of renal cytosols from *L. elliptica* collected in an austral summer is shown in fig. 1a. The elution profile showed that about 90 % of the Cd eluted occurred between 90 and

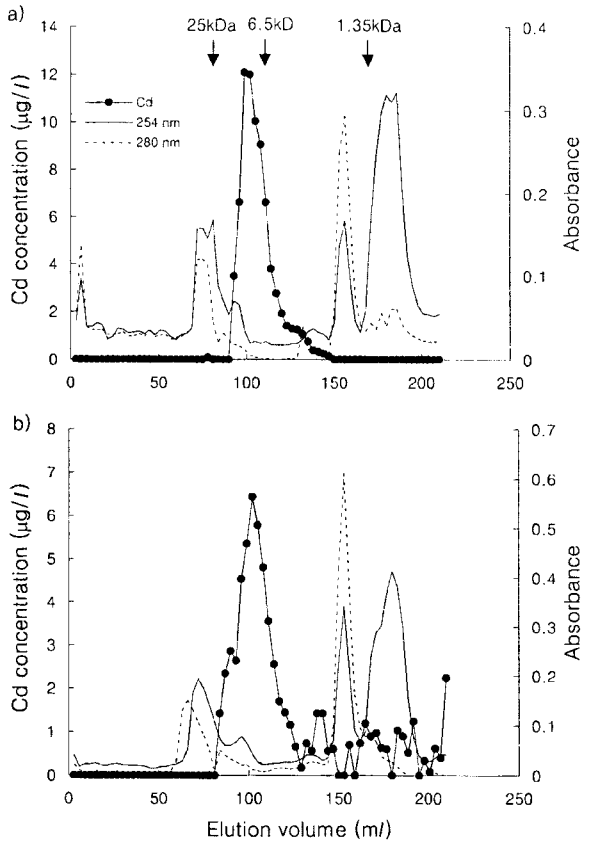

Fig. 1. Elution profiles of Sephacryl S-100 chromatography of *Laternula elliptica* renal cytosol collected in an austral summer (a) and in a winter (b).

110 m*l* fractions with a peak at about 100 m*l* fraction. This corresponds to the apparent molecular weight of about 10-12 kDa. The elution profile of Cd for cytosol of winter samples showed a pattern similar to the profile of summer samples, indicating high Cd concentration in the fractions collected between 90 and 120 m*l* (Fig. 1b). The fraction number that showed the maximum concentration of Cd in the eluent of the winter samples also coincided with that of the summer samples. About 65 % of the total Cd eluted was associated with proteins eluted in the fractions collected between 90 and 120 m*l*.

The Cd containing fractions collected from gel filtration chromatography were pooled and subjected to further purification on a DEAE ion exchange chromatography and the ratios of Cd to protein determined for each purification step are shown in Table 2. The gel filtration chromatography resulted in about 23-fold increase in purification from the initial total homogenate. However, the DEAE anion exchange chromatography did not increase the purification from the gel filtration step. The polyacrylamide gel electrophoresis demonstrated that soluble high molecular weight proteins were removed from the cytosol by gel-filtration and DEAE ion exchange chromatography (Fig. 2). After the ion exchange chromatography, the Cd-containing fractions contained proteins of apparent molecular about 10 kDa, 18 kDa, and 24 kDa.

Table 2. Cadmium/protein ratios of Cd-binding protein fractions of *Laternula elliptica* kidney obtained during the purification stage.

Sample	Cd/protein	Relative purification
Total homogenate	5.3×10^{-4}	1 - fold
Cytosol	3.1×10^{-3}	5.9 - fold
Cd-binding fraction (Sepahcryl S-100)	1.2×10^{-2}	22.7 - fold
Cd-binding fraction (DEAE ion exchange)	0.9×10^{-3}	17.1 - fold

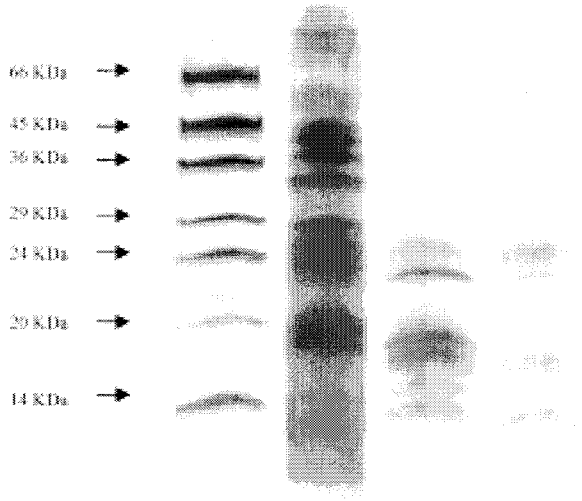

Fig. 2. SDS-PAGE of *Laternula elliptica* kidney cytosol (lane 2), and fractions containing metallothionein-like Cd-binding protein after Sephacryl S-100 chromatography (lane 3) and DEAE ion exchange chromatography (lane 4). Protein standards of different molecular weight are shown in lane 1.

Location of metallothionein in the kidney

Location of MT in the *L. elliptica* kidney was examined with immunochemical staining with a commercial monoclonal mouse MT antibody (DAKO). Intense reactivity to the mouse MT-antibody was detected at the outer stripe of the epithelial cells of the kidney (Fig. 3), indicating the presence of MT in the epithelial cells. The shapes of the

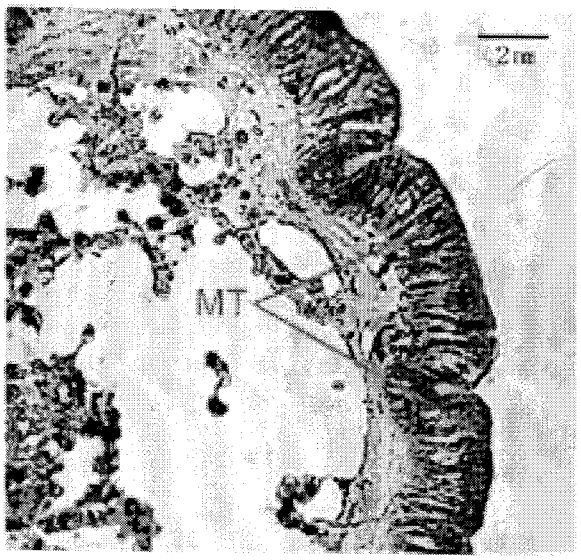

Fig. 3. Light micrographs of immuno-reactivity against monoclonal mouse MT antibody in the epithelial cells of *Laternula elliptica* kidney. The section was counter stained with hematoxylin.

epithelial cells appeared to be columnar.

4. Discussion

Cadmuim concentration in the kidney

The kidney is the major site for metal accumulation in molluscs. Strong accumulation of Cd, up to five orders of magnitude higher than the concentrations in the environment (Lee *et al.* 1990; Bargagli *et al.* 1996), was shown in the kidney of *Larternula elliptica* in this study. The Cd concentrations in the kidney total homogenate are comparable to the values found in the kidney or viscera of other molluscs exposed to unnaturally high Cd concentrations (Cassini *et al.* 1986; Bebianno and Langston 1995; Roméo and Gnassia-Barelli 1995; Serra *et al.* 1995; Isani *et al.* 1997), although they are closer to the lower limit of the Cd concentration range measured in the kidney of *L. elliptica* in previous studies (Ahn *et al.* 1996, in press; Nigro *et al.* 1997).

Strong accumulation of metals in the mollusc kidney is mainly due to effective sequestration of metals within the kidney concretions or granules in particulate fraction (George *et al.* 1980; Sullivan *et al.* 1988; Marigómez *et al.* 1990; Nott *et al.* 1993) and to metal-binding proteins in the cytosolic compartment in cells (Viarengo *et al.* 1985; Mason and Jenkins 1995; Bebianno and Langston 1998). In the kidney of *L. elliptica*, both the particulate and cytosolic compartments play important roles in Cd sequestration (Table 1). Elution profiles of chromatography indicate that there exists an important Cd-binding protein in the cytosol of kidney that was associated with most of the Cd eluted (Fig. 1). Cadmium sequestration to the particulate fraction was important as much as to metal binding proteins in the cytosolic fraction (Table 1) and this is in contrary to the results shown in other Cd exposure studies on various mollusc species. When the animals were exposed to high concentrations of Cd in a short period of time, most of the Cd was sequestered in cytosolic fractions containing metal binding proteins (Langston and Zhou 1987a,b; Bebianno and Langston 1992; Hylland *et al.* 1994; Roméo and Gnassia-Barelli 1995). Experimental factors such as the length of exposure time and the exposure concentration are important in determining the subcellular distribution of metals (Amiard-Triquet *et al.* 1998; Hamza-Chaffai *et al.* 1999). The shown difference in subcellular distribution of Cd in *L. elliptica* and other experimental results might have come from the difference in exposure conditions. Different from the animals exposed to high concentrations of Cd in short time interval, the slow growing *L. elliptica* are chronically exposed to Cd for their life time which can reach more than 20 years at it maximum shell length of >100 mm (Brey and Mackenson 1997). A similar result was reported from the Antarctic scallop *Adamusium colecki*, of which the accumulation of Cd occurs for a long period time as in *L. elliptica*. In the digestive gland, the organ that showed highest Cd accumulation, about 70 % of the Cd was associated with the particulate fractions (Viarengo *et al.* 1993). At present stage, we have not identified what are the major binding ligands of Cd in the particulate fraction of *L. elliptica* kidney. Electron dense concretions were observed in the particulate fractions microscopically (data not shown). A future study is required to characterize these concretions and also to clarify their roles in Cd sequestration.

A metallothionein-like protein in the cytosol

The results of this study demonstrate that a Cd-binding protein is present in the cytosol of *L. elliptica* kidney and this protein has characteristics of MTs found in a wide variety of temperate molluscs. The apparent molecular weight of the fractions where Cd was mainly eluted from gel filtration chromatography, which is about 10-12 kDa (Fig. 1), coincides with the size of MTs found in other molluscs by size exclusion chromatography (Duquesne *et al.* 1995; Serra *et al.* 1995; Bordin *et al.* 1997; Engel

1999; Ponzano *et al.* 2001). It has been generally known that MTs have low spectral absorbance at 280 nm due to the absence of aromatic amino acids and relatively higher absorption at 254 nm (or 250 nm) with the presence of metal(Cd)-thiolate bonds in their composition (Kimura *et al.* 1979; Johansson *et al.* 1986; Viarengo *et al.* 1988; 1993; 1999; Duquesne *et al.* 1995; High *et al.* 1997). The Cd-binding protein detected in this study by the gel filtration chromatography also showed relatively low absorbance at 280 nm (Fig. 1) indicating that the protein has similar characteristics of MTs. However, the absorbance peak at 254 nm was relatively low and occurred slightly earlier than the retention time of the Cd peak (Fig. 1), probably because the Cd-binding MT-like protein eluted was not completely separated from other proteins of similar sizes. Detection of MTs by UV absorbance is sometimes technically difficult for natural populations (Lacorn *et al.* 2001) and a distinct absorbance peak at 254 nm related with MTs was detected only after the samples were treated with several purification steps (Olafson *et al.* 1979; Paris-Palacios *et al.* 2000; Lacorn *et al.* 2001). Electrophoresis results show that the Cd-containing fractions obtained from gel filtration and ion exchange chromatographies are actually composed of several different sizes of proteins (Fig. 2). Nonetheless, a protein with molecular weight of about 10-12 kDa, which is thought to be an MT-like protein is one of the major bands of the Cd-containing fraction. Further research on the amino acid composition and structure is needed to confirm this protein is actually an MT. Strong immune reactivity to an MT antibody detected at the kidney epithelial cells also supports that there exists an MT-like protein in the kidney of *L. elliptica* (Fig. 3).

Though not clearly identified as an MT in this study, the MT-like Cd-binding protein is present in the kidney of *L. elliptica* both in the summer and in the winter and may play a key role in Cd sequestration and detoxification as indicated by the result showing that most of the Cd eluted was associated with this protein (Fig. 1). Other prerequisites for a protein is to be effective as a detoxifying agent are that its synthesis must be sufficiently rapid and binding to metals should be strong enough (Langston *et al.* 1998). Future studies on the inducibility of the Cd-binding MT-like protein upon exposures to excessive Cd will clarify the role of this protein in Cd detoxification in the kidney of *L. elliptica*.

Location of MT-like protein in the *L. elliptica* kidney

Presence and importance of MTs in metal accumulation in the kidney of molluscs have often been examined biochemically (Roesijadi 1980; Fowler & Gould 1988; Evtushenko *et al.* 1990; Bebianno & Langston 1995; Duquesne *et al.* 1995; Serra *et al.* 1995). However, the exact location of these proteins in the mollusc kidney has never been confirmed. Our study result suggests that the outer stripe of the epithelial cells are the major site for MT in *L. elliptica* kidney as indicated by the strong reactivity to a mouse MT antibody in these cells (Fig. 4). To our best knowledge, this is the first report that microscopically demonstrated the location of MT or MT-like protein in the mollusc kidneys. Earlier studies observed only metal-rich granules or concretions in the epithelial cells of the kidney of marine molluscs (Geroge *et al.* 1980; Mason *et al.* 1984; Simkiss & Mason 1984; Nigro *et al.* 1992; Viarengo & Nott 1993). Despite the high accumulation of Cd in association with the MT-like protein, no signs of damages were shown in the epithelial cells. The shape of the cells was columnar as in other healthy bivalve mollusc kidneys (Pierie and George 1979; George *et al.* 1980), supporting the idea that the MT-like protein plays an important role in the detoxification of Cd in the kidney of *L. elliptica*.

Acknowledgements

The authors appreciate Dr. S.H. Lee and K.W. Cho for their careful reading of the manuscript and providing valuable comments. This work was supported by the Korea Science and Engineering Foundation(No. R05-2000-00004) and Korea Ocean Research and Development Institute.

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Received Oct. 6, 2001
Accepted Dec. 17, 2001