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Cadmium-Inducible Metallothionein in Tilapia (*Oreochromis mossambicus*)

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Metallothioneins (MTs), containing over 33% cysteine content and lacking both aromatic amino acids and histidine, are metal binding proteins with low molecular weight (7-14 kDa). The metal ions are bound exclusively through thiolate coordination complexes which involve all 20 cysteine residues (Dunn et al. 1987). The MTs can be induced by and bind metals such as cadmium, copper, mercury, silver and zinc (Roesijadi 1992) and are capable of binding 6-7 atoms of cadmium or zinc per mole (Kägi and Schaffer 1988; Hamer 1985). The metals bound to MTs can be removed by exposure to low pH. MTs exist in vertebrates, aquatic invertebrates (Roesijadi 1992), certain bacteria (Olafson et al. 1988) and fusion yeast (Hamer et al. 1985). These proteins are found most abundant in liver, kidney, pancreas and intestine of animals, and the expression of these proteins showed a dose-response relation with the degrees of heavy metal pollution. Expression of MTs has also found to be induced by steroid hormones, interferons, certain chemicals, hepatotoxic solvent, and stresses (Kägi and Schaffer 1988). MTs have been implicated in the involvement of metabolism of copper and zinc (Cousins 1985), and in detoxification of pollutant metals, such as cadmium and mercury (Kägi and Kojima 1987). Moreover, MTs have been indicated to be an efficient scavenger for free $OH^{\cdot}$ (Dunn et al. 1987).

Heavy metals are only found at low concentrations in the aquatic environment. However, they are fairly common pollutants of mining, smelting, plating, second-hand metal recovery operations and other anthropogenic activity. The toxicity of heavy metals to aquatic fishes, their accumulation within the tissues and the impact on the physiological mechanisms has also well studied (Mance 1987; Heath 1987). In aquatic organisms, the chemical, molecular, cellular physiological and toxicological properties of MTs have been recently studied as a specific and potential biomarker for metal pollution in aquatic environment (Sampath-Kunar et al. 1996; Yeoh et al. 1996; Brower et al. 1995; Gerorge et al. 1996). Moreover, the detoxification of MT was suggested to be one of the major factors to affect the tolerance of heavy metals in aquatic animals (Roesijadi 1992).

Metal-binding protein or MT has been purified and characterized from several species of teleost, including skipjack *Katsuwonus pelamis* (Takeda and Shimizu

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1982), plaice *Pleuronectes platessa* (Overnell and Coombs 1979), carp (Kito et al. 1982) and catfish *Heteropneustes fossilis* (Chatterjee and Maiti 1987). However, the amino acid sequence has not been determined from the purified MT in these species. Most of the amino acid sequence published so far was deduced from cDNA sequence (Kille et al. 1991; George et al. 1990; Bonham et al. 1987). In Asia, tilapia is one of the most common and widespread species inhabiting rivers and estuaries. Tilapia is not only an important aquaculture species but also one of the most popular model species for toxicological research. However, only a few papers so far discussed the subject of MT in tilapia. The relation of cortisol and expression of MT-like protein was investigated in tilapia (Fu et al. 1990). Ueng et al. (1996) determined the content of cytosolic MT by cadmium-hemoglobin and silver-saturation methods and suggested that MT is existing in tilapia, but they have not studied any biochemical character of the native tilapia MT. Recently the cDNA sequence and the mRNA expression of MT are found in tilapia (Chan 1994), but no any data related to the MT protein is available in his study. The present study was focused on the identification of MT from tilapia (*Oreochromis mossambicus*) after cadmium induction in order to demonstrate that tilapia MT is responsive to inductive effect of cadmium.

MATERIALS AND METHODS

Mature tilapia (*Oreochromis mossambicus*) from the Tainan Branch of the Taiwan Fisheries Research Institute were reared in circulating freshwater at 26-28°C and under a photoperiod of 12-14 hr lighting. Tilapia were immersed in water containing 6-7 mg/L cadmium for 13-14 d or injected with 0.2 mg $CdCl_2$/kg body weight every 2 d for 3 doses. The tissues were taken out immediately and stored at -70°C until analyzed.

Tissues were weighed, dried at 37°C overnight, and then digested in 13.1 N HNO_3 at room temperature overnight. Digests as well as water samples of incubation media were diluted with double-deionized water and cadmium (Cd) was analyzed by an atomic absorption spectrophotometer (Z-8000, Hitachi, Japan), using air/acetylene flame and graphite for Cd^{2+} analysis. The Cd standard solution (Merck, Germany) was used as a standard curve to calculate the Cd contents in unknow samples.

The procedure of purification was that previously described (Overnell and Coombs 1979; Kito et al. 1982; Takeda and Shimizu 1982; Chatterjee and Maiti 1987; Richards and Steele 1987; Richards 1989; Liebrich et al. 1995) with some modifications. Liver of Cd-immersed tilapia was dissected and homogenized in homogenization buffer (10 m*M* Tris-HCl buffer, 5 m*M* 2-mercaptoethanol, pH 8.6) in 4 volumes of tissue weight. The homogenization was centrifuged at 15,000xg for 40 min. The supernatant was heated at 65°C for 10 min, cooled and ultracentrifuged again at 40,000xg for 1 hr. The supernatant was loaded on a Sephadex-G75 column (Pharmacia, 8 cm x 2.5 cm, 4°C ; elution buffer 10 m*M* Tris-HCl, 0.02% NaN_3, pH 8.6) at a flow rate of 0.7 ml/min. The elution was monitored UV detector (254 nm), and fractions were collected. The MT-

containing fractions were determined directly by the Cd content through atomic absorption spectrophotometer. The molecular weight of native Cd-containing protein was determined by separating protein standards according to the procedure above (Sephadex G75). The protein standards were albumin (MW 67,000), ovalbumin (MW 43,000) and ribonuclease A (MW 13,700). Partition coefficients (kd) were evaluated from the relationship $kd = (V_e-V_0)/(V_t-V_0)$. All Cd-containing fractions were pooled and loaded onto a DEAE-Sephadex A25, anion exchange (25 cm x 2 cm column), and the column was eluted with a Tris-HCl linear gradient (from 250 m*M* Tris-HCl to 600 m*M* Tris-HCl; flow rate 0.7ml/min). The elutions were collected and dialyzed with double distilled water in 3,500 MW dialysis bag for 36 hr (changed every 12 hr for 2 times). After lyophilization, the pellet was dissolved in 1 mL double distilled water for HPLC.

A high performance liquid chromatography apparatus equipped with L 6000 dual pumps, a computer system controller (L 6200, Hitachi, Japan), a column (RCM-C18, 8 cmx10 mm, Waters, U.S.A) and a data module (Macintosh, U.S.A) was used to perform the separation and quantitation of MT. Buffer A consisted of 10 m*M* sodium phosphate (pH 7.0), and buffer B consisted of 80% acetonitrile (ACN) plus 20% 10 m*M* sodium phosphate . MT was eluted with a three-step gradient, which consisted of 0-5% buffer B for 0-5 min and run at flow rate of 1 ml/min, followed by 5-20% buffer B from 5-10 min and the last step was 20-25% buffer B from 10-25 min at flow rate of 2 ml/min. The elution was monitored UV-VIS detector (L4200, Hitachi, Japan) at 254 nm wavelength. The thiol group was measured spectrophotometrically with dithio-2-nitrobenzoic acid (DNTB) (Sigma, U.S.A) as described by Ellman (1959).

A low molecular weight SDS-PAGE system (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was used (Schagger 1987). The gel (12 x 1.5 cm) was run at 80 mA constant current for 4 hr. The gel was stained with coomassie brilliant blue, and the MW marker was purchased from Gibson (peptides 3-43 kD). One gel was stained with coomassie blue R250 and was destained with 10% acetic acid and 50% methanol, and then transferred to PVDF (polyvinylidene difluoride) membrane at 20 mA for 16 hr. The transferred membrane was cut into small slices and incubated with 70% formic acid containing 70 m*M* Cyanogen Bromide (CNBr) for 24 hr to delete the methylated N-terminal of polypeptide. Then, the amino acid sequence of the CNBr-cleaned MT was analyzed by 10 cycles of Edman degradation with a pulsed-liquid protein sequencer (477A Applied Biosystem , U.S.A) equipped with an on-line phenyl-thiohydantoin analyzer (120A, Applied Biosystem, U.S.A) and a Macintosh IIsi computer. Determination of cysteine in tilapia MT was performed by the method described by Hsu (1982).

RESULTS AND DISCUSSION

Tilapia, which were injected with 0.2 mg $CdCl_2$/kg body weight every 2 d for 7 d or immersed in 6-7 mg/L $CdCl_2$ for 14 d, revealed various relative amounts of Cd (%) in different organs including liver, kidney, pancreas, intestine, testis and

Table 1. Relative amounts (%)[a] of cadmium accumulated in different organs of tilapia (*Oreochromis mossambicus*) injected with or immersed in $CdCl_2$ solution.

Organ	Treatment	
	Injection[b] (N=5)	Immersion[b] (N=8)
Liver	38.86±6.4[c]	23.6±5.3[c]
Kidney	13.24±2.3	11.4±7.1
Stomach	6.66±l.l	8.1±3.4
Intestine	27.444±4.1	29.1±10.9
Gill	3.20±0.4	9.9±4.5
Testis	4.48±0.4	3.4±1.6
Spleen	5.28±0.3	5.2±4.3
Muscle	0.95±0.1	[d]

[a] Relative amount = each organ/ all the organs studied× 100%
[b] Tilapia were immersed in 6-7 mg/L $CdCl_2$ for 14 d or injected with 0.2 mg/kg $CdCl_2$ every 2 d for 3 doses
[c] Mean±SD was indicated
[d] Undetected

Table 2. Recovery rate of the cadmium and protein content from the liver of cadmium-treated tilapia after homogenization and extraction.

Treatment	Cadmium			Protein		
	Homogenate (μ mole/g)	Extraction[b] (μ mole/g)	Recovery Rate[c] (%)	Homogenate (mg/g)	Extraction[b] (mg/g)	Recovery rate[c] (%)
Injection[a]-1	340	0.6	0.18	94.41	0.46	0.5
Injection[a]-2	400	0.9	0.23	98.85	0.65	0.1
Immersion[a]- 1	7000	14.0	0.20	89.03	13.89	14.7
Immersion[a]-2	2390	7.7	0.32	90.28	9.82	9.9

[a] Tilapia were immersed in 6-7 mg/L $CdCl_2$ for 14 d or injected with 0.2 mg $CdCl_2$ /kg body weight every 2 d for 3 doses. 1 and 2 represent the experiment repeated twice. The value present the pool of 5 tilapias
[b] After centrifugation
[c] Calculated from the ratio of extraction and homogenate

gill. The distribution of accumulated Cd in organs was different among studies. In the studies by injection was showed liver > intestine > kidney; by immersion was intestine > liver >kidney (Table 1), as in Woo et al. (1993) paper. After short time exposure to 0.5 mg/L $CdCl_2$, the data showed higher Cd accumulation in kidney (1180 ppb) than that in liver (126 ppb) and gill (5 ppb). However, in the case of 1 mg/L Cd exposure, the Cd accumulation in gill (31 ppb) was higher than that in liver (17.5 ppb). Some previous studies also reported that relative amounts of Cd accumulation were kidney> liver> gill (George 1996; Cattani et al. 1996), or liver>kidney > gill >intestine (Chatterjee and Maiti 1987). The inconsistency

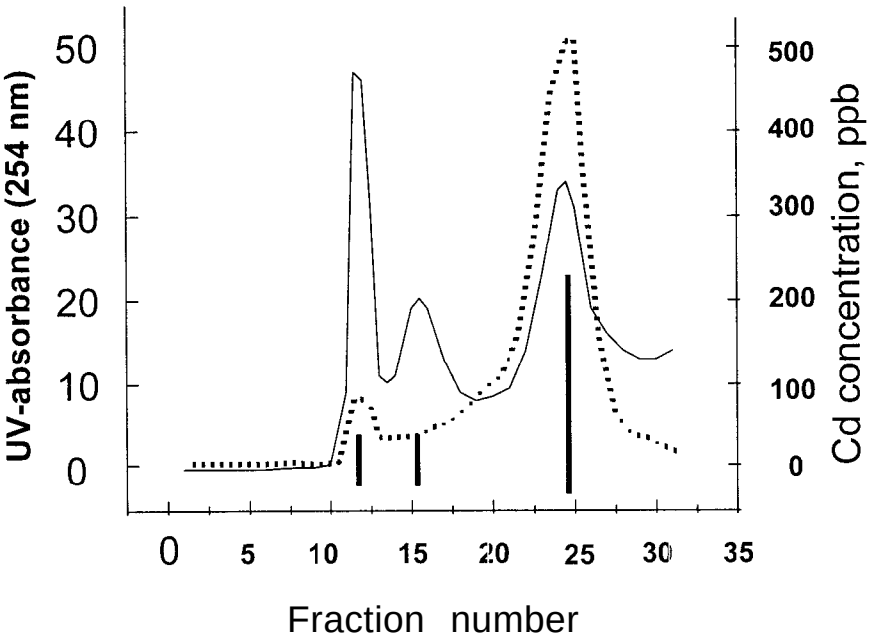

Figure 1. Elution of Cd-treated tilapia liver homogenate on a Sephadex G-75 column. Dotted line represents the Cd content of fractions (μg/ml). Solid line presents the absorbance at 254 nm. Thin bar stands for the thiol group.

between the present results and previous studies may be ascribed to the differences in (a) dose and exposure time of Cd (b) method of administration (c) species and (d) environment. In our preliminary experiments the recoveries of Cd and protein were higher in the case of immersion than those in the case of injection. Therefore, liver from the tilapia immersed in Cd was chosen for MT purification. The recovery of Cd and protein in liver after homogenization and centrifugation was 0.2-0.3% and 10-14%, respectively (Table 2). The supernatant of liver homogenization was applied to Sephadex G75 gel filtration (Fig. 1). The profile of the chromatograph showed 3 major peaks, and the highest level of Cd appeared in the 3rd peak. The thiol group was coincident with the peak of Cd level. The thiol group of the 3rd peak was over 7-10 times higher than this of the first and second peaks (Fig. 1). This result suggests that the major Cd peak contained the highest content of MT. It was known that treatment with Cd would induce the synthesis of MT-like protein (Fu et al. 1990). MT is generally considered as a "housekeeping" protein because it is expressed in many different tissues and cell type (Hamer 1986). Moreover, high Cd concentrations in tissues of Cd treated animals could easily be explained by enhanced levels of metallothionein-bound cadmium (Liebrich 1995). The elution volume of the major Cd peak was between ovalbumin (MW 43,000) and albumin(MW 67,000), and the V_e/V_0 was 2.07. The kd (partition coefficient) value of tilapia MT in gel filtration was similar to the data reported previously, 1.8-2.2. The molecular weight of native tilapia MT was estimated about 54 kDa. Therefore, we suggested that the MT forms a polymer which stores in tilapia. Indeed, MTs are generally present in polymer forms in

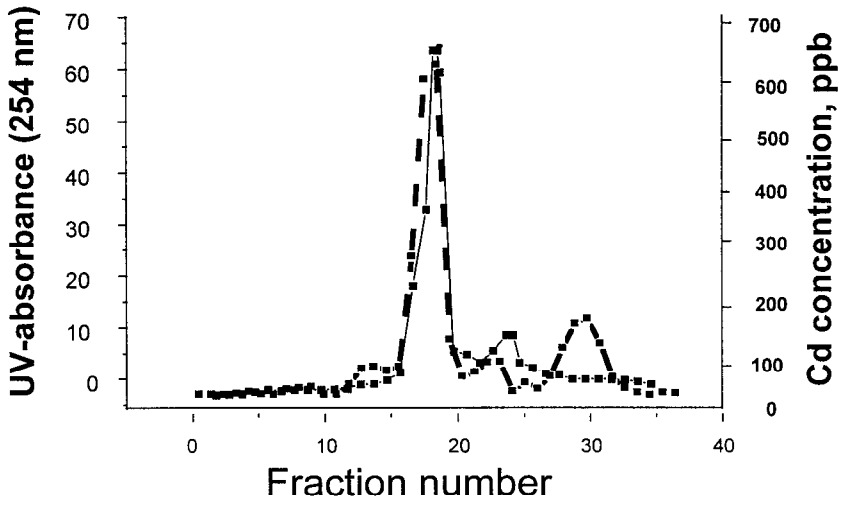

Figure 2. The elution of metallothionein fraction obtained from gel fraction on DEAE Sephadex A-25. The separation was conducted with a linear gradient, 250-600 m*M*, of Tris-HCl buffer (pH 8.6) at 4°C. Dotted line represents the Cd content of fractions (μg/ml). Solid line presents the absorbance at 254 nm.

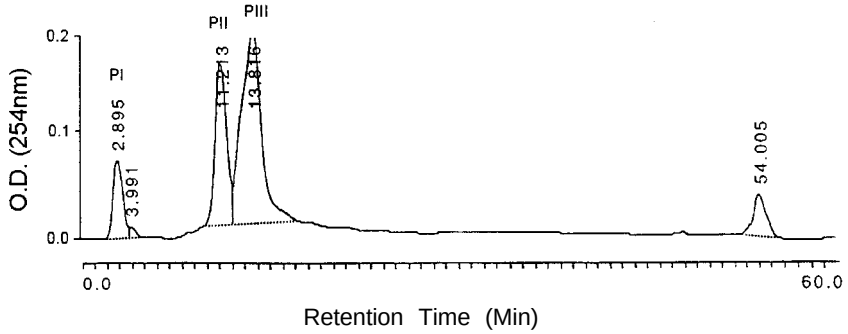

Figure 3. The separation of tilapia metallothionein on a RP-HPLC

animals (Templeton and Chenan 1984).

The pooled fractions were subjected to DEAE-Sephadex A25 chromatography, and two peaks of Cd-bound components were eluted with 399.5 m*M* land 443.2 m*M* of Tris-HCl, respectively (Fig 2), indicating that tilapia MTs would be elutedat high ionic concentrations in ion exchange. In previous studies, differentsolutions (NaCl or Tris-HCl) were used for MT elution, and 130 m*M* was generally used for the elution of MT (Kito et al. 1982; Chatterjee and Maiti 1987;Richards and Steele 1987). There were 3 major peaks after RP-HPLC

Table. 3. Changes of cadmium (μg/L) in 3 peaks of HPLC before and after filtrationn by cut-off filter*.

	Cadmium	
peak	Before cut-off	After cut-off
P1	1065	10.0
P2	84	45.6
P3	160	68.1

* The filtration was processed by the 5 kD MW ultrafilter.

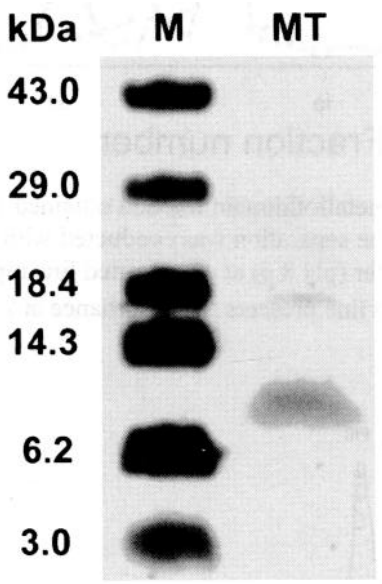

Figure 4. SDS-PAGE of the third peak of HPLC from tilapia liver sample. The gel was stained with coomassie brilliant blue. Lane 1: protein markers, Lane 2: p3 of HPLC.

separation (Fig. 3). The first peak (p1), which was eluted at 2.9 min, contained most free Cd and less protein. The second (p2) and third (p3) peaks were separately eluted at 11.2 and 13.8 min and contained 84 and 160 ppb Cd, respectively (Table 3).

Moreover, the thiol group was higher in p3 than that in p2 (data not shown). In the present study tilapia MT was eluted at 25% of ACN. However, most previous studies showed that MT I and MT II could be eluted at the ACN concentration which was gradually increased to 16% (Richards and Steele 1987; Richards 1989;Liebrich et al. 1995), and MT III was at 37% ACN (Chen et al. 1996). Considering higher Tris-HCl with higher CAN in elution, tilapia MT may be more electronegative than that in mouse, rat, chicken or hamster. And tilapia MT being an hydrophilic protein may have a higher polarity than other fish or animal MTs. Purified tilapia MTs were subjected to SDS-PAGE to determine the molecular

weights in denatured condition. All samples showed at least 2 bands, and their MW was 6.2 and 18.2kDa (Fig 4.). According to tilapia MT cDNA (Chan 1994) and based on our SDS-PAGE results, we suggest that the molecular mass of MT can be estimated about 7 kDa in monomer or 14 kDa in dimer. Chatterjee and Maiti (1987) also suggested that Mt is present as dimers and tetramers in catfish (*Heteropneustes fossslis*) based on the molecular weight, 24-28 kDa on SDS-PAGE gels. Moreover, monomeric, dimeric and trimeric forms of MTs were found in rat and dogfish after 2-mercaptoethanol treatment (Hidalgo et al. 1988). The results suggest that effect of 2-mercaptoethanol on the molecular weight (MW) of MT is eliminated the MT oligomeric forms and consequently promoting the existence of a low-molecular-weight protein band on SDS-PAGE gels. The MW of MT is 10-12 kDa in dab *Limanda limanda* L (Kammann et al. 1996), 13-15 kDa in plaice and 11 kDa in rock fish (Kito et al. 1982). Indeed, purified MTs in different previous studies were inconsistent in molecular weight. The differences in the molecular weights and in the gel results among different studies suggested that the mobilization of MTs on SDS-PAGE is affected the linkage of metals (Dunn et al. 1987).

Comparing the p2 and p3 obtained from HPLC, the Cd levels and thiol group of p2 were much lower than those of p3. However, the SDS-PAGE of p2 and p3 showed the similar results. Several previous studies have reported over two peaks in purification and suggested the presence of different isoforms of MT (Richards and Steele 1987; Kito et al. 1982; Mackay 1993). Whether p2 and p3 in the present study are isoforms of tilapia MT needs to be further studied. The p3 of HPLC was collected and reacted with CNBr to cut out the first methylated amino acid. The amino acid sequence of CNBr-reacted MT was determined as DPCECAKTG. Compared with the nucleic acid sequence of tilapia MT cDNA (Chan 1994) the data showed over 90% similarity between amino acid and nucleic acid sequences. The amino acid sequence of tilapia MT was highly homologous to those of other fishes. Obviously, we are the first to confirm the purified MT by using the pulsed-liquid protein sequencer to directly delineate the amino acid sequence of the purified tilapia MT. In most previous studies, the procedures for purification of MT are generally included: extraction, gel filtration, ion exchange and high performance liquid chromatography. Furthermore, SDS-PAGE was generally applied to determine MW, and amino acid composition analysis was conducted to confirm the purified MT by the determination of cysteine. In some other metal-binding proteins, such as superoxide dismutase, alcohol dehydrogenase and alkaline phosphatase, were also found to bind metals (Saito and Kojima 1997). Results of amino acid compositions may not exclude some possible mistake when the purified MT is contaminated with some other metal-binding proteins. Conclusively, the present study implies that the inducible MT which is confirmed by purification, characterization and sequencing is the response of heavy metal i.e. Cd in tilapia.

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