[Frontiers in Bioscience 11, 2113-2122, September 1, 2006]

Development of ELISA for metallothionein-II allows determination of heavy metal pollution of fresh water

Ming-ming Chu [1], Zhen-quan Guo [1], Norio Muto [2], Norio Itoh [2], Keiichi Tanaka [2], and Hong-wei Ren [1]

[1] National Laboratory of Protein Engineering and Plant Genetic Engineering, College of Life Sciences, Peking University, Beijing 100871, China, [2] Department of Toxicology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamada-oka, Suita, Osaka 565-0871, Japan

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1. ABSTRACT

Metallothionein (MT), a metal-binding protein induced primarily by heavy metals in vertebrates, is considered a biomarker for environmental heavy-metal contamination. To investigate heavy metal pollution in the freshwater environment, MT-I and MT-II were purified from livers of crucian carp (*Carassius carassius*) by gel exclusion chromatography and ion exchange chromatography. To detect the purified MT-II, a specific monoclonal antibody (mAb) against crucian carp MT-II was produced from the hybridoma strains by cell-cell fusion. By using Enzyme-Linked Immunosorbent Assay (ELISA) with this mAb, the purified crucian carp MT-II was detected with a high specificity and sensitivity. There was a good correlation between the amount of MT-II in carp livers and the concentration of heavy metals in water. ELISA was then used to evaluated the degree of heavy metal pollution in two freshwater systems. The results indicate that the MT-II content in carp liver tissue can be used as an indicator of environmental heavy-metal pollution.

2. INTRODUCTION

Metallothionein (MT), a low molecular weight, metal-binding protein comprised of about 60 amino acids, exists exclusively in a variety of animal species, plants and microorganisms (1). MTs are characterized by a high cysteine content (about 30%), absence of aromatic amino acids and histidine and a high-affinity for heavy metals (2, 3). MT-I and MT-II are distributed in various tissues and specific organs, including liver and kidney. MTs function in (i) regulation of the essential trace metals, such as copper and zinc, (ii) scavenging of free-radicals, and (iii) detoxification of excess amounts of heavy metal elements (cadmium, mercury, copper and zinc) (4). Organisms that express MTs resist heavy metal toxicity. MT-deficient yeast and mice, for example, are more sensitive to metal toxicity than wild types (5-7). Several metal responsive elements (MRE) and glucocorticoid responsive elements (GRE) exist in the promoter region of MT-I and MT-II genes, which can be strongly induced by heavy metals and weakly induced by hormones (8-12).

Contamination in the environment and organisms can be quantified through analytical chemistry. However, the analytical results do not necessarily reflect biological responses to metal exposure. Analysis of MT content in aquatic animals is being used as a bio-indicator to evaluate environmental pollution of heavy metals (13, 14).Several methods can be used to detect MTs in aquatic animals, including metal binding assays (15, 16), polarography (17), molecular probes (18), RT-PCR (19, 20) and immunological assays (21, 22). Immunological assays, such as Enzyme Linked Immunosorbent Assay (ELISA), have been used to detect low levels of MTs in aquatic animals (23). Compared with other methods, immunological assays are simple, rapid, sensitive and suitable for field applications: they are particularly effective for detecting a range of environmental pollution (24).

A practical immunological assay for MTs in fish was previously developed in our laboratory (25). MT content in fish was determined by ELISA with rabbit polyclonal antibodies. Analysis of MT content in different fish species from different source waters indicated that (i) liver and kidney were the two main organs that accumulate heavy metals, and (ii) MT levels rose when the metal concentration of water increased. In this study, we generated a monoclonal antibody (mAb) against crucian carp (C*arassius carassius*) MT-II, which can recognize MT-II in liver tissue of crucian carp and some other teleosts. Using ELISA, we investigated MT-II content in the livers of crucian carp from two fresh water areas (GaoBeiDian Lake and YuYuanTan Park) in Beijing. Concentrations of heavy metals in water were determined by atomic absorption spectroscopy (AAS). Data from the immunological assay indicate that variations in MT-II content reflect levels of heavy metals in freshwater.

3. MATERIALS AND METHODS

3.1. Apparatus and chemicals

Metal analysis was performed by AAS using a conventional dual-option burner system (Philip PU-9200). A pH meter equipped with a glass electrode combined with an Ag/AgCl reference electrode was used for pH measurements. An ultraviolet (UV) absorption detection with 254 nm and 280 nm wavelength was employed for metallothionein detection and purification. Cadmium chloride ($CdCl_2$), magnesium chloride ($MgCl_2$), mercaptoethanol, sodium azide, polyethylene glycol (PEG) 2000, 3, 3-diaminobenzidin tetrahydrochloride (DAB), hypoxanthin and thymidine (HT) and hypoxanthin, aminopterin and thymidine (HAT) additives were obtained from Sigma Chemical Co.. Dulbecco's modification of Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco/BRL Co.. Peroxidase-conjugated rabbit antibodies to rat immunoglobulin and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin were obtained from Beijing Zhongshan Biotechnology Inc. (Beijing, China). Duck MT-II, rabbit MT-II and mouse MT-II were prepared in our laboratory.

Crucian carps were obtained from Dazhongsi Fish Farm (Beijing, China). Balb/c mice were purchased from the Beijing Animal Center (Beijing, China).

3.2. Purification of crucian carp liver MT-II

Crucian carps of 15-20 cm in length from the Dazhongsi Fish Farm were maintained in two tanks (150 cm length×50 cm width×50cm depth) for two weeks. Air was gently bubbled into each tank to maintain oxygen levels. To enhance MT-II protein concentrations, the abdominal cavities of the fish were injected with $CdCl_2$. On the 1^{st}, 4^{th}, 7^{th} and 10^{th} days, fish were injected with 2mg/kg ($CdCl_2$/fish weight), 4mg/kg, 6mg/kg and 8mg/kg respectively. On day 11, fish were sacrificed to obtain livers.

The livers were weighed and homogenized in 10 mM Tris-HCl buffer (pH8.6, containing 2 mM mercaptoethanol and 10 mM sodium azide; about 50 g livers in 75 ml Tris-HCl buffer) with a vitreous homogenizer. After centrifugation at 106,000×g at 4^oC for 60 minutes, the supernatant was heated at 80^oC for three minutes and centrifuged at 10,000×g at 4^oC for three minutes. The supernatant was then subjected to gel filtration on a G-50 column (2.6×120 cm) pre-equilibrated with 10 mM Tris-HCl buffer (pH 8.6, containing 2 mM mercaptoethanol and 10 mM sodium azide) at room temperature. Each fraction identified by gel exclusion chromatography was quantified for Cd (II) atomic absorption, 254 nm and 280 nm UV absorption.

The fractions with high Cd (II) atomic absorption and 254 nm UV absorption were loaded on a DEAE-Sepharose column (2.6×15 cm) pre-equilibrated with the Tris-HCl buffer for ion exchange chromatography. The loaded column was equilibrated with 10 mM Tris-HCl (pH 8.6, containing 2 mM mercaptoethanol and 10 mM sodium azide) to remove uncombined proteins. Cadmium-binding MTs were eluted with a linear gradient of Tris-HCl buffer (pH 8.6), ranging from 10 to 500 mM (A buffer, 10 mM Tris-HCl; B buffer, 500 mM Tris-HCl, pH 8.6), and monitored by Cd (II) atomic absorption, 254 nm and 280 nm UV absorption. The fractions with high Cd (II) atomic absorption and 254 nm UV absorption were combined and the 2^{nd} peak (MT-II protein) (25) was loaded on a G-25 column (1.6×120 cm) pre-equilibrated with 10 mM NH_4HCO_3 (containing 2 mM mercaptoethanol). The high 254 nm UV absorption fraction was collected and frozen at -20^oC. The proteins were subjected to SDS-PAGE. The sulfydryl content was detected using Ellman's reagent as described previously (26).

3.3. Production and identification of mAb against crucian carp MT-II

The mAb against crucian carp MT-II was prepared by the hybridoma technique. Briefly, purified crucian carp MT-II (200 μg) in 500 μl phosphate buffered solution (PBS) was mixed with 500 μl Freund's complete adjuvant for each Balb/c mouse. The mixture was injected into the peritoneal cavity of one Balb/c mouse followed by three booster immunizations in four weeks with 100 μg MT-II in 200 μl PBS mixed with Freund's incomplete adjuvant. After the final injection, a small amount of blood was gathered from the tails of immunized mice and analyzed by ELISA. Twenty μg MT-II in 100 μl PBS was then injected directly into the spleen of each Balb/c mouse that

demonstrated high immunological competence to further stimulate an immunological response. After three days, spleen cells from immunized mice were fused with mouse plasmacytoma cells (Sp2/0) at a ratio of 10:1 with 50% PEG 2000. The hybridomas were then cultured in 96-well flat-bottomed cell culture plates with 200 μl culture of DMEM containing 20% FBS, 100 μM hypoxanthin, 0.4 μM aminopterin and 16 μM thymidine (HAT medium) together with mouse peritoneal cavity cells as feeder cells. The hybridomas were allowed to expand for about one week. The HAT medium was changed every third day. Aminopterin was then withheld from DMEM, which contained 20% FBS, 100 μM hypoxanthin and 16 μM thymidine (HT medium).

Two weeks after hybridization the supernatant of each cell culture well was analyzed with ELISA, in microtiter plates coated with 1 μg MT-II per well. The positive cultures were cloned by limited dilution. Colonies from a single cell were further expanded and characterized by ELISA. The positive colonies from a single cell were injected into the peritoneal cavities of Balb/c mice and after about ten days, ascites were gathered for subsequent experimentation.

Ascites were purified by caprylic acid and saturated ammonium sulfate solution (SAS). Ascites (1 ml) were slowly mixed for 30 minutes with 4 ml 0.06 M NaAc-HAc solution (pH 4.8, containing 125 μl caprylic acid). The mixture was centrifuged at 10,000×g at room temperature for 30 minutes. The supernatant was mixed with 500 μl 0.1M PBS (pH 7.4), cooled to 4^{o}C, then combined with 1.6 ml SAS (pH 7.4) , while gently moving the mixing container in a circular pattern. After two hours at 4^{o}C□the mixture was centrifuged at 10,000×g at 4^{o}C for 30 minutes. The pellet was redissolved in 4 ml PBS (pH 7.4) and stored at -20^{o}C. The mAb was detected by SDS-PAGE and Western blotting.

3.4. Characterization of the mAb against crucian carp MT-II

The isotype of the mAb in culture medium was determined by ELISA. Peroxidase-conjugated rabbit antibodies to rat IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgM and IgA were used as secondary antibodies for isotype analysis. The affinity constant of the mAb was calculated as described by Borrebaeck C *et al.* (27).

MT-II from crucian carp (*Carassius carassius*), duck (*Mergus merganser*), rabbit (*Oryctolagus cuniculus*) and mouse (*Mus musculus*) was detected by ELISA and western blotting for cross reaction. The tissues of crucian carp, common carp (*Cyprinus carpio*), grass carp (*Ctenopharyngodon idellus*) and zebrafish (*Brachydanio rerio*) including livers, kidneys, gills and muscles were weighed and homogenized in 10 mM Tris-HCl buffer (pH8.6, containing 2 mM mercaptoethanol and 10 mM Sodium Azide; 1 mg tissues in 10 ml Tris-HCl buffer) with a vitreous homogenizer. After centrifugation at 106,000×g at 4^{o}C for 30 minutes, the supernatant was heated at 80^{o}C for three minutes and centrifuged at 10,000×g at 4^{o}C for three minutes. The supernatant was analyzed by ELISA and western blotting.

3.5. Detection of crucian carp MT-II and metal concentrations in two fresh water lakes

YuYuanTan Park and GaoBeiDian Lake were chosen to collect crucian carps, because major urban areas of Beijing city are located between them. Crucian carps (15~20 cm) were caught with barbs and taken to our laboratory where they were sacrificed by freezing. The livers of 20 crucian carps (6 from GaoBeiDian Lake, 8 from YuYuanTan Park and 6 from water without any metal as a control) were removed, weighed and homogenized in 10 mM Tris-HCl buffer (pH8.6, containing 2 mM mercaptoethanol and 10 mM Sodium Azide; 1 mg livers in 10 ml Tris-HCl buffer) with a vitreous homogenizer. After centrifugation at 106,000×g at 4^{o}C for 30 minutes, the supernatants were heated at 80^{o}C for three minutes and centrifuged at 10,000×g at 4^{o}C for three minutes. A 100 μl aliquot of each supernatant was coated onto a microtiter plate for the ELISA test.

Surface samples of water from GaoBeiDian Lake and YuYuanTan Park were collected into clean bottles and submitted for quantification of trace metals using atomic absorption photometry. Two ml 100 mg/L $MgCl_2$ were added to a 250 ml water sample, followed by 2 ml 200 mg/L NaOH; the mixture was swirled during the addition to ensure a homogenous solution. The treated sample was allowed to sit, undisturbed for approximately two hours. Settled material was dissolved with 1 ml 50% HNO_4 and then added to 25 ml final volume by purified water (i). A standard solution of five (mixed) metals was prepared by combining 1.5% HNO_4 with 1.0 mg/L cadmium, 2.0 mg/L copper, 2.5 mg/L iron, 2.5 mg/L zinc and 5.0 mg/L lead. Purified water was respectively added into 0, 1.0, 2.0, 3.0, 4.0, 5.0 ml of the standard solutions to 250 ml final volumes (ii). Water samples (i) and the standard solutions of 6 grads (ii) were detected by flame atomic absorption photometry (FAAS). Working condition: air flow rate, 5.5 L/min; air pressure, 0.15 MPa; acetylene flow rate, 0.6 L/min; acetylene pressure, 0.04 MPa; lamp current, 8 mA; working wavelength, 324.8 nm (copper), 283.3 nm (lead), 228.8 nm (cadmium), 259.9 nm (iron) and 213.8 nm (zinc); burner height, 1.5 mm; spectrum bind-width, 1 mm. The concentrations of the five metals were calculated by using FAAS standard curves.

3.6. Statistical analysis

Results are expressed as means (± S.E.M.). Excel (Microsoft Office 2003) was used to identify significant differences with t-tests at a 95% confidence level ($p<0.05$). Relationships between different crucian carp MT-II concentrations and corresponding OD_{450} values were initially examined in bivariate scatterplots and tested by simple correlation (Pearson's r, $y=ax+b$).

4. RESULTS

4.1. Identification of crucian carp MT-II

Based on different isoelectric points of crucian carp MTs, MT-I (peak 1) was first eluted from DEAE Sepharose Fast Flow column and then MT-II (peak 2) (Figure 1). Crucian carp MTs indicated high Cd (II) atomic absorption and 254 nm UV absorption, but low 280 nm UV absorption, since MTs combine with Cd (II) and are

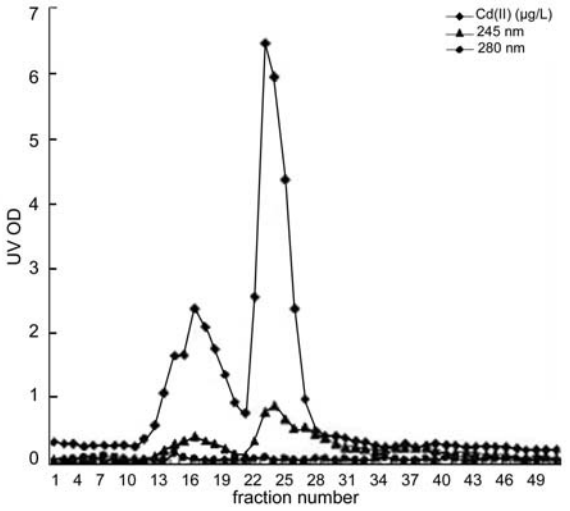

Figure 1. Ion exchange chromatography (DEAE Sepharose Fast Flow) of pooled fractions from gel filtration. Column size: 2.6×15cm, Eluted with the linear gradient of Tris-HCl buffer (A buffer, 0.01 M Tris-HCl; B buffer, 0.5 M Tris-HCl, pH 8.6). Flow rate is 90ml/h, 10ml/tube. Peak A, crucian carp MT-I; Peak B, crucian carp MT-II.

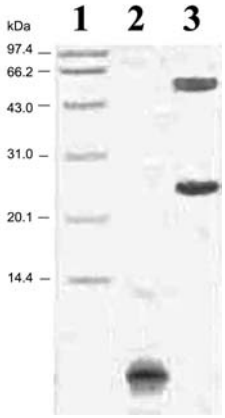

Figure 2. SDS-PAGE of crucian carp MT-II. Lane 1: protein standards; Lane 2: purified crucian carp MT-II (7 kDa); Lane 3: mAb against crucian carp MT-II (heavy chain, 50 kDa; light chain, 25 kDa).

characterized by a high cysteine concentration and absence of aromatic amino acids and histidine. About 16 mg crucian carp MT-II protein with a molecular weight of approximately 7,000 was extracted from 50 g livers (Figure 2). The SH group content was 29.3% by Ellman's reagent, in accordance with MTs' naming principle. These data indicate that MT-II obtained had the characteristics of common MTs.

4.2. Identification and characterization of mAb against crucian carp MT-II

After cell fusion, nine wells of cells (1F4, 2E6, 1E10, 1D6, 1F10, 1C4, 2F7, 1F6, 2G7) that secreted positive antibodies were detected, amplified and stored in liquid nitrogen. One well (1E10) was selected for subcloning. After subcloning three times, seven wells (B3, B4, B6, C3, C5, D2, F6) were found to contain monoclonal secreting strains. These positive monoclonal cells were injected into the peritoneal cavities of ten Balb/c mice and 20ml ascites were gathered. After purification, the mAb with a molecular weight of about 150 kDa (Figure 2) could specially recognize crucian carp MT-II protein instead of MT-II from duck, rabbit and mouse tissues by ELISA and western blotting (Figure 3A and Figure 3B). The mAb could recognize native MT-II from related teleost fishes such as cyprinoid and grass carp but not zebrafish (Figure 4A). This difference emphasizes the genetic similarities among some species. However, the mAb only recognized denatured crucian carp MT-II by western blotting; it did not react with denatured MT-II of cyprinoid and grass carp (Figure 4B), thus emphasizing that some genetic differences do exist even among species in the same family. The isotype of the mAb was IgG_1 by the ELISA test (Figure 5). The functional affinity constant was $(2.61\pm0.29)\times10^9$ M^{-1} by noncompetitive ELISA, based on the following formula (Figure 6).

Functional affinity constant = (n-1)/2(n[Ab']-[Ab])

n is the multiple of antigen concentrations coated in microtiter plates ([Antigen]/[Antigen']), and

[Ab'] and [Ab] are the corresponding antibody concentrations.

It was assumed that the mAb could be combined with two MT-II protein molecules.

The concentration of mAb that nearly combined with all corresponding antigens was chosen for ELISA as the best concentration. From the ELISA curves shown in Figure 6, the best concentration of the purified mAb was 10^6 ~10^7 ng/L.

4.3. Study of heavy metal pollution in Beijing freshwater environment

Lead, zinc, cadmium and iron concentrations in GaoBeiDian Lake water were much higher than those in YuYuanTan Park water (Table 1). Copper, on the other hand was higher in water from YuYuanTan Park. Compared with international water quality standards, only copper and iron concentrations in YuYuanTan Park water exceeded the standard by 400% and 150%; lead, cadmium and zinc concentrations were within the acceptable range. In GaoBeiDian Lake water, on the other hand, lead, cadmium and iron concentrations exceeded the standard by 150%, 140% and 760%, respectively. Zinc and copper concentrations were not considered excessive. While copper and iron are necessary trace elements in living organisms and play important biochemical roles, lead and cadmium are generally considered nonessential and toxic. Based solely on water concentration data, GaoBeiDian Lake is more heavily polluted than YuYuanTan Park. Lead and cadmium couldn't be detected in YuYuanTan Park water. The specific sources of the heavy metals in GaoBeiDian Lake were not investigated. However, in a large city such as Beijing, there are numerous point and nonpoint sources that contributed to environmental pollution, including heavy metals.

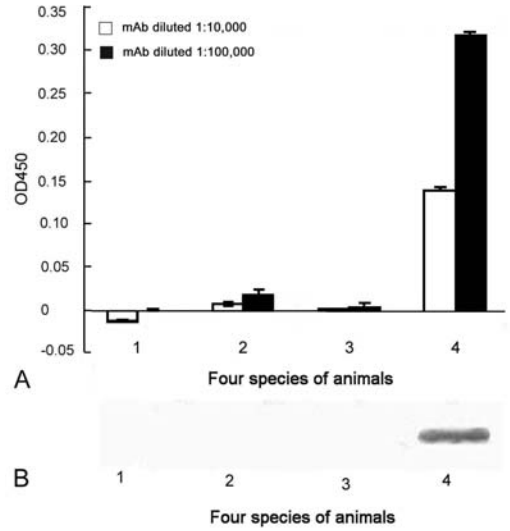

Figure 3. Cross reactivity of the mAb against crucian carp MT-II with MT-II from four species of animals. A. ELISA of rabbit (1), mouse (2), duck (3) and crucian carp (4) MT-II using mAb against crucian carp MT-II. MT-II/PBS (1 μg/100 μl) was coated in microtiter plates. The mAb against crucian carp MT-II (1:10,000 diluted, black bar; 1:100,000 diluted, open bar) was used for the first antibody, horseradish peroxidase-conjugated goat anti-mouse immunoglobulin for the second antibody. Only crucian carp MT-II showed a positive result (rabbit, mouse and duck indicated statistical differences from the crucian carp, t-test, $p<0.01$). B. Western blotting of rabbit (1), mouse (2), duck (3) and crucian carp (4) MT-II. The mAb against crucian carp MT-II and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin were used for the first and second antibodies respectively, with the substrate of DAB. Only crucian carp MT-II (line 4) showed a positive result.

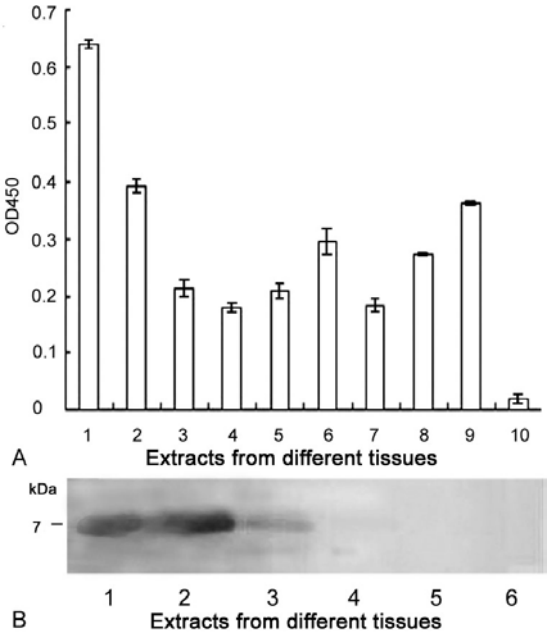

Figure 4. Differences in MT-II from crucian carp, common carp, grass carp and zebra fish by immunoassay. A. ELISA of tissue extracts from four species of native fishes. 1, 1 μg purified crucian carp MT-II/100 μl PBS; 2~5, 100 μl 1:10 diluted tissue extracts from crucian carp livers, kidneys, gills and muscles; 6~9, 100 μl 1:10 diluted tissue extracts from common carp livers and kidneys, grass carp livers and kidneys; 10, 100 μl 1:10 diluted tissue extracts from zebra fishes. Crucian carp, common carp and grass carp tissue extracts all showed positive results, however zebra fish extracts appeared negative (zebra fish indicated statistical differences from others, t-test, $p<0.01$). B. Western blotting of tissue extracts from four species of fishes. Respectively, 1 μg purified crucian carp MT-II (1, positive bar), 10 μl tissue extracts from crucian carp livers induced by Cd (II) (2), native crucian carp livers (3), native common carp livers (4), native grass carp livers (5) and native zebra fishes (6). The mAb against crucian carp MT-II and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin were used for the first and second antibodies respectively, with the substrate of DAB. Tissue extracts from crucian carp livers induced by Cd (II) (2) indicated a higher MT-II content than that from native crucian carp livers (3) based on their different bars. Tissue extracts from common carps (4), grass carps (5) and zebra fishes (6) showed negative results.

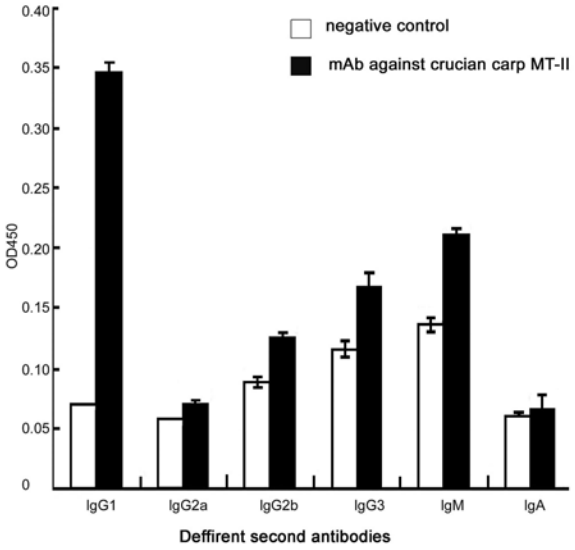

Figure 5. Isotype analysis of the mAb against crucian carp MT-II. Purified crucian carp MT-II (1 μg) was coated in microtiter plates by ELISA with first antibody of the mAb against crucian carp MT-II and second antibody of peroxidase-conjugated rabbit antibodies to mouse IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgM and IgA. 20% HT medium (open bar) replaced the first antibody (mAb against crucian carp MT-II, black bar) as the negative control. The final effective result was OD (black bar) − OD (open bar). IgG_1 indicated statistical differences from others, t-test, $p<0.01$).

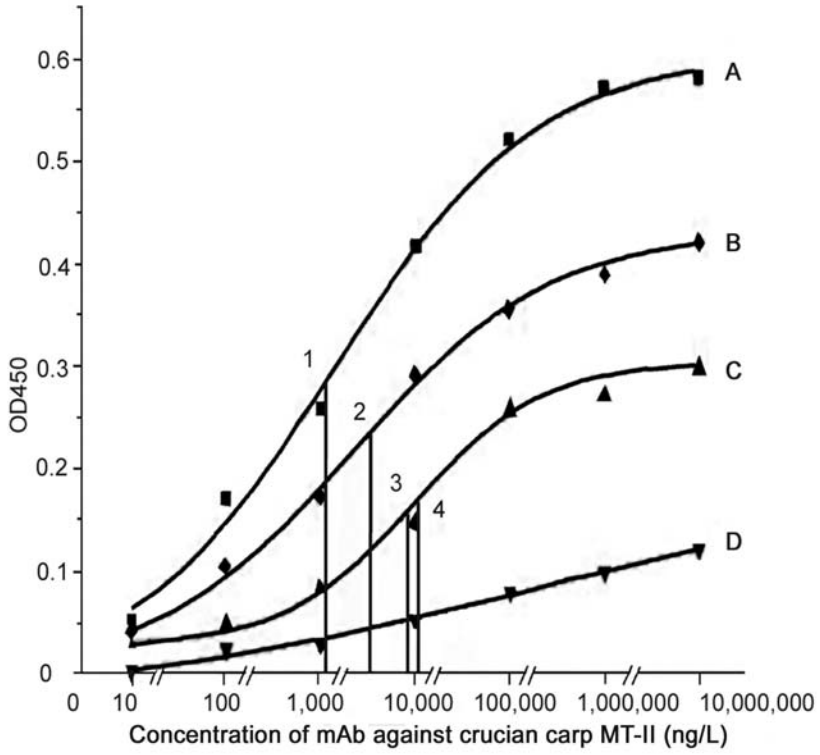

Figure 6. Affinity constant curves of crucian carp MT-II and the mAb against crucian carp MT-II. Four concentrations of crucian carp MT-II, 1 μg/100 μl, 0.5 μg/100 μl, 0.25 μg/100 μl and 0.125 μg/100 μl(MT-II/PBS) (A, B, C and D in curve respectively) were coated in microtiter plates. They were detected by ELISA with the first antibody of the mAb against crucian carp MT-II diluted differently. Where OD_{450} value was 50% of the maximal OD_{450} value, the corresponding mAb concentrations were 1.5×10^3, 6.0×10^3, 9.5×10^3 and 1.2×10^4 ng/L (curves 1, 2, 3 and 4 respectively). Based on the formula and the functional affinity constant = (n-1)/2(n[Ab']-[Ab]) (see text), six affinity constants were calculated to be 2.30×10^9, 2.38×10^9, 2.47×10^9, 2.57×10^9, 2.76×10^9, 3.17×10^9 M^{-1}, the average value was $2.61\pm0.29\times10^9$ M^{-1}.

Table 1. Water concentrations of five metals from YuYuanTan Park and GaoBeiDian Lake

Water source	Lead	Copper	Zinc	Cadmium	Iron
International water quality standard	<0.1	<0.05	<5.0	<0.01	<0.1
Water from YuYuanTan Park	ND (DL=0.001)	0.2041±0.0028*	0.0548±0.0031**	ND (DL=0.001)	0.1537±0.0026***
Water from GaoBeiDian Lake	0.1550±0.0025	0.0375±0.0029*	0.1247±0.0018**	0.0145±0.0034	0.7675±0.0036***

Values are mg/L, expressed as means ± S.E.M. ND means concentrations measured below the detection limit (DL). (*), (**) and (***) indicate statistical differences between YuYuanTan Park and GaoBeiDian Lake (t-test, *p<0.01, **p<0.01 and ***p<0.01).

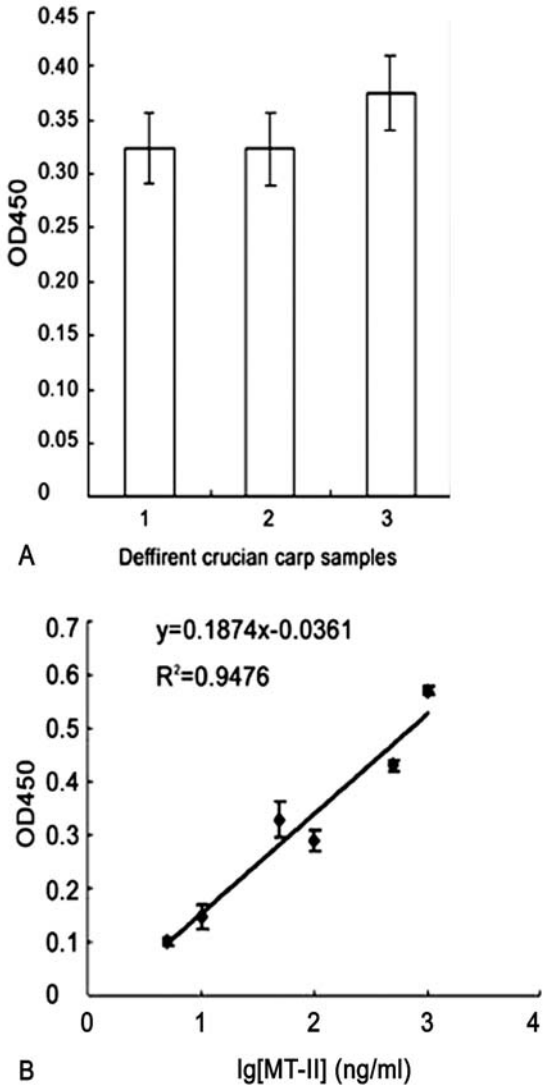

Figure 7. A. ELISA of liver MT-II of crucian carps in GaoBeiDian Lake and YuYuanTan Park. 1~3, 100 µl 1:10 diluted liver extracts from common crucian carps in laboratory (control, n=6), crucian carps from YuYuanTan Park (n=8) and crucian carps from GaoBeiDian Lake (n=6). The concentration in GaoBeiDian Lake fish was significantly higher than the concentration from YuYuanTan Park (t-test, p<0.05) and the laboratory (t-test, p<0.05), which were not different from each other. B. Standard curve for MT-II determination by ELISA. MT-II concentrations of crucian carp liver extracts from YuYuanTan Park, GaoBeiDian Lake and the laboratory were 82.22 ng/ml, 155.96 ng/ml and 82.22 ng/ml respectively.

Crucian carp liver MT-II contents from GaoBeiDian Lake and YuYuanTan Park are shown in Figure 7. The data show increasing MT-II levels in fish from GaoBeiDian Lake. Based on the standard curve, the MT-II concentration of crucian carp from GaoBeiDian Lake was 155.96 ng/ml, nearly double of the concentration from YuYuanTan Park and the laboratory (82.22 ng/ml).

5. DISCUSSION

Recently, several animal species such as the wild bank vole (28), *Lithognathus mormyrus* (19), mosquito *Culex quinquefasciatus* (29) and fish (30-33) have been considered as biological models to determine environmental heavy metal pollution. Since crucian carp (*Carassius carassius*) is a common teleost in many limnetic rivers and lakes, and is easy to obtain, development of a specific and sensitive immunoassay system would be useful for monitoring heavy metal pollution in fresh water.

MTs are easily induced by heavy metals because of the metal responsive elements in the promoter region of MT genes (8, 9). Some studies indicate the expression level of MT-II is very low under base conditions, while its mRNA increases dramatically in response to Cd exposure (34). These findings show that MT-II is more sensitive to Cd exposure than MT-I, and therefore, MT-II is the preferred choice for an indicative protein. Unlike mammalian MTs, polyclonal antibodies of crucian carp MT-II can only recognize MT-II, not MT-I (unpublished data). N-terminal sequencing indicates that, unlike MT-I, the N-terminal of crucian carp MT-II is unblocked by an acetyl group (35). Consequently, existence of MT-I does not affect detection of MT-II in immunoassays.

Using ELISA, the mAb against crucian carp MT-II can recognize native MT-II from crucian carp (*Carassius carassius*), common carp (*Cyprinus carpio*) and grass carp (*Ctenopharyngodon idellus*). The antibody does not react with MT-II from zebrafish (*Brachydanio rerio*) (Figure 4A). The three carp species are from the same family (*Cyprinidae*) and share the same antigenic determinant reactive to the mAb. However, the mAb can detect the denatured MT-II only from crucian carp (Figure 4B). This suggests that amino acid sequences of these three teleosts were slightly different at the antigenic level. Moreover, some studies show that metal uptake from the dissolved phase, and the concentration of MT in an organism, are significantly dependent on the body size (36, 37). Although crucian carp possess the smallest body size of the three carp species, this species had the highest MT-II concentration (Figure 4). Therefore, the combined efficiency of mAb against crucian carp MT-II with common carp and grass carp MT-II is lower than with crucian carp MT-II. The mAb is only useful for quantitative detection of crucian

carp MT-II. Furthermore, levels of MT-II are greatly different in various tissues (36, 38, 39) (Figure 4A). For monitoring purposes, concentration of MT should be measured in fish liver tissues, rather than in kidney or gill tissues, because the liver MT is more sensitively induced than in other tissues (40).

Multiple methods have been developed over the last two decades for quantification of MTs in biological tissues. A cadmium-haemoglobin affinity assay was developed in 1982 as a general method for rapid determination of MTs in biological tissues (41). This method can detect about 40 ng MTs, but can be complicated by the presence of Ag+ and Cu^{2+}. Thus, a silver-saturation method was developed in 1986 for detection of MTs (42). However, many low molecular-weight proteins in tissues can combine with metals and confound results. Capillary electrophoresis (CE) is an efficient method for separating proteins based on broad-range electrolytes, but the most suitable capillary and separating conditions need to be chosen carefully (43). Although the recently developed RT-PCR method can detect MTs in the transcription level (19, 20), it cannot determine the change of MTs from mRNA to protein. Moreover, because of fast degradation of mRNA, it is difficult to apply this method to researches outside of the laboratory. Another method, polarography, depends greatly on specific and delicate instrumentation and has limited applicability. Compared with these other methods, immunoassay presents distinct advantages. Based on the specific recognition reaction between antigen and antibody, an indicator protein can be easily quantified with a high degree of resolution. Immunoassay, such as ELISA, represents a more commercially viable product, based on the stability of the protein (antibody) and detecting reagents. Immunoassay can meet the requirements of most situations and is a sensitive way to rapidly detect the target protein.

The different MT-II contents of crucian carp collected from YuYuanTan Park and GaoBeiDian Lake were correlated with the concentrations of heavy metals from the two water sources. It is reasonable to assume that the concentrations of MT-II in carp is carp is in direct response to exposure to metals in the water. Therefore, the results reported here are consistent with evidence presented by other researchers that MTs can be used as sensitive biomarkers of heavy metal contamination (30, 44, 45).

6. ACKNOWLEDGEMENTS:

This work was supported by Chinese National Sciences Fund Committee (NSFC. Grant No. 96-C02-01-09 and 20420160084).

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Abbreviations: MT: metallothionein; ELISA: Enzyme-Linked Immunosorbent Assay; mAb: monoclonal antibody; PBS: phosphate buffered solution; FBS: fetal bovine serum; DMEM: Dulbecco's modification of Eagle's Medium; SAS: saturated ammonium sulfate solution.

Key Words: Metallothionein, heavy metal pollution, ELISA, monoclonal antibody

Send correspondence to: Dr Hong-wei Ren, the National Laboratory of Protein Engineering and Plant Genetic Engineering, College of Life Sciences, Peking University, Beijing 100871, China, Tel: 86-10-62755800, Fax: 86-10-62751842, E-mail: renhw@pku.edu.cn