



HAL
open science

Quantitative immunochemical evaluation of fish metallothionein upon exposure to cadmium

Yana Yudkovski, Adelina Rogowska-Wrzesinska, Irena Yankelevich, Edna Shefer, Barak Herut, Moshe Tom

► **To cite this version:**

Yana Yudkovski, Adelina Rogowska-Wrzesinska, Irena Yankelevich, Edna Shefer, Barak Herut, et al.. Quantitative immunochemical evaluation of fish metallothionein upon exposure to cadmium. *Marine Environmental Research*, 2008, 65 (5), pp.427. 10.1016/j.marenvres.2008.02.001 . hal-00563000

HAL Id: hal-00563000

<https://hal.science/hal-00563000>

Submitted on 4 Feb 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Quantitative immunochemical evaluation of fish metallothionein upon exposure to cadmium

Yana Yudkovski, Adelina Rogowska-Wrzesinska, Irena Yankelevich, Edna Shefer, Barak Herut, Moshe Tom

PII: S0141-1136(08)00017-2
DOI: [10.1016/j.marenvres.2008.02.001](https://doi.org/10.1016/j.marenvres.2008.02.001)
Reference: MERE 3169

To appear in: *Marine Environmental Research*

Received Date: 24 November 2007
Revised Date: 1 February 2008
Accepted Date: 1 February 2008

Please cite this article as: Yudkovski, Y., Rogowska-Wrzesinska, A., Yankelevich, I., Shefer, E., Herut, B., Tom, M., Quantitative immunochemical evaluation of fish metallothionein upon exposure to cadmium, *Marine Environmental Research* (2008), doi: [10.1016/j.marenvres.2008.02.001](https://doi.org/10.1016/j.marenvres.2008.02.001)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



Quantitative immunochemical evaluation of fish metallothionein upon exposure to cadmium

Yana Yudkovski ^a, Adelina Rogowska-Wrzesinska ^b, Irena Yankelevich ^a, Edna Shefer ^a, Barak Herut ^a, Moshe Tom ^{a*}

^a Israel Oceanographic and Limnological Research, P.O.B 8030 Haifa, 31080 Israel.

^b University of Southern Denmark, Institute for Biochemistry and Molecular Biology, Campusvej 55, 5230, Odense-M, Denmark

* Corresponding author. Address: Israel Oceanographic and Limnological Research, P.O.B. 8030, Haifa, 31080, Israel.
Telephone: 972-4-8565257, Telefax: 972-4-8511911, E - mail: tom@ocean.org.il

Abstract

Efficient implementation of an environmental biomarker requires multi-annual comparability over a wide geographical range. The present study improved the comparability of a quantitative competitive metallothionein (MT) Enzyme-Linked-Immuno-Sorbent-Assay (ELISA) in the sentinel fish *Lithognathus mormyrus* by introducing to the assay recombinant MT and beta-actin standards. Commercial antibodies for cod MT and mammalian actin were implemented. In addition, a sensitive anti *L. mormyrus* MT antibody was produced, adequate only for solid phase immunochemical assays. Cadmium was applied to the fish through injection and feeding to serve as a testing platform of the ELISA. The results demonstrated high potential protective capacity of the liver against toxic levels of transition metals through increasing MT levels. MT transcript levels were evaluated also from fish sampled at polluted and relatively clean natural sites, indicating applicability of MT as biomarker of exposure to a multi-factorial pollution, in comparison to its low revealed sensitivity to controlled cadmium exposure.

Keywords

Biomarker; metallothionein; beta-actin, *Lithognathus mormyrus*; Mediterranean; real time PCR; ELISA; cadmium, biomonitoring

1. Introduction

Levels of environment-affected transcripts and proteins, evaluated in a native sentinel species are widely used as environmental biomarkers of an examined habitat. The appropriate establishment of diagnostic environmental gene-product biomarkers in general and in fish was recently discussed in Handy et al. (2003), Van der Oost et al. (2003) and Tom and Auslander (2005). The latter review defined long-term comparability of biomarker levels over a wide geographical range as an essential requirement from an environmental biomarker. Evaluations done by more than one laboratory would further complicate the comparisons. The suggested components of an environmental bio monitoring and its characteristics include: (1) standard proteins or transcripts, enabling absolute quantification of the biomarker levels. Recombinant products are preferred, securing supply and repeatability of the standards. (2) Normalization using constitutively expressed gene products which are processed concurrently with the target biomarker and evaluated by similar methods. (3) The range of the sub-lethal biomarker values have to be determined experimentally, scaling the levels obtained from feral sentinels within the attainable range.

A series of studies performed in our laboratory (Tom et al. 2002, 2003, 2004, Funkenstein et al., 2004) used the above guidelines to adapt two generic methods to be implemented as environmental biomarker assays, using the striped sea bream (*Lithognathus mormyrus*) as sentinel fish. Quantitative real time PCR was used for transcripts evaluations and Enzyme-Linked-Immuno-Sorbent-Assay (ELISA) for proteins. Both methods were modified to include appropriate specific transcript or protein standards compatible with the target fish. Specific PCR primers and anti-protein antibodies were used as detection probes.

The striped sea bream was selected as sentinel fish of its native sandy coastal habitat as it is widely distributed throughout the entire Mediterranean and also in the South-Eastern Atlantic (Bauchot and Hureau, 1986). It is a bottom-dwelling fish, inhabits polluted and pristine coastal sandy habitats and interacts with the water column but also with the bottom, through its bottom-feeding habits and burial behavior (Suau, 1970). Its gut usually contains sediment, engulfed with

the ingested food (Froggia, 1977; Buxton et al., 1984; Lasiak, 1984). It is a protandrous hermaphrodite (Besseau, 1990; Kraljevic et al., 1995), similar to other members of its family, the *Sparidae* (Buxton and Garratt, 1990; Micale and Perdichizzi, 1994). The gonad is an ovotestis, sexually indistinguishable outside the summer reproductive season. In addition, there are also no external sex-distinguishing characters (Bauchot and Hureau, 1986; Kraljevic et al., 1995).

Metallothionein (MT) is a 6-7 kDa metallo-sulphur rich protein (Hamer, 1986; Kagi, 1991). Hepatic MT synthesis is induced by a number of metals, cytokines and stress hormones as well as by a wide range of chemicals, many of which act indirectly via a stress or inflammatory response (Coyle et al., 2002). Its transcript and protein are induced in fish by a variety of bi-valent metals, including cadmium, zinc, copper, lead and mercury and were widely used as biomarkers for evaluating the cumulative biological effect of transition metals disposed to the marine environment (Chan et al., 1989; George and Olsson, 1994; George et al., 1996; Olsvik et al., 2001; Tom et al., 1999, 2004; Atli and Canli, 2003; De Boeck et al., 2003; Eroglu et al., 2005; Long and Wang, 2005; Woo et al., 2006; Alvarado et al., 2006; Hansen et al., 2006).

Transition metals are found in the marine environment as free ions, as well as in a variety of complexes with suspended particles and sediments. Fish are exposed to the various transition metal species at different intensities. There are two major routes of metals exposure. Metal ions dissolved in the ambient water are absorbed through the gills (Alvarado et al., 2006) and other permeable body surfaces. Metals bound to solid particles are ingested, detached from their carrier particles in the digestive system and absorbed through the gut epithelium (Berntssen et al., 2001).

Four parameters were basically used to evaluate MT protein levels: MT metal content, metal substitution, the amount of MT sulfhydryl groups and MT immunochemical affinity (Hylland, 1999). A competitive ELISA was described by Hylland (1999) using a native purified cod MT as standard protein and anti-cod MT antibody. Wu et al. (1999, 2000) described similar ELISA for tilapia using partial synthetic MT as coating buffer and the native purified protein as standard. The major aim of the present study was to adapt this competitive ELISAs to *L. mormyrus* and its

improvement by introducing recombinant MT as standard. Parallel ELISA of beta-actin was developed to be used as normalizing agent, applying recombinant beta-actin as standard. A by-product of this study was the production of sensitive anti *L. mormyrus* MT antibody. Unfortunately, this antibody reacted only with MT immobilized on solid phase, therefore, was not adequate for our competitive ELISA.

In vitro production of recombinant MT in *E. coli* was carried out in both mammals and fish (Vergani et al., 2003; Yang et al., 2007). Beta-actin was *in vitro* produced in mammals using Sf9 insect cell host and baculovirus vector (Joel et al., 2004).

Exposure of *L. mormyrus* to food-borne and injected cadmium was served as platform for testing the two developed ELISAs. It was used also to simultaneously measure hepatic levels of MT transcript and protein as well as the corresponding levels of bio-accumulated cadmium in relation to the applied metal ions. In addition, hepatic MT transcript levels were measured in feral fish sampled at two natural habitats of *L. mormyrus* and served to compare realistic environmental situation to controlled exposure to a single pollutant.

2. Material and Methods

2.1. General molecular methods

General biochemical and molecular protocols followed Sambrook and Russell (2001) and include RNA electrophoresis, spectrophotometric evaluation of RNA concentrations, plasmid manipulations and selective growth of bacteria on Luria-Bertani (LB) medium-agar plates. PCR assays, excluding real time PCR, were done in final concentrations of 0.05 units μl^{-1} Taq DNA polymerase, in buffer provided with the enzyme. The solution also contained 0.2 mM of each d-nucleotide triphosphate, and primer concentrations of 10 μM . PCR designs and applied templates are detailed below for each specific reaction.

2.2. Synthesis of recombinant MT

The synthesis generally followed Vergani et al (2003) with several modifications. The coding region of *L. mormyrus* MT (GenBank Accession no. AF321007) was modified by PCR (95°C – 2 min; 35 cycles of [95°C -1 min, 51°C – 1 min, 72°C – 1 min]; 72°C -2 min) to include EcoRI and XhoI restriction sites at its two ends. The MT cDNA-containing vector served as PCR template, applying the primer pair 5'-TCCCCGGAAT TCATGGACCC GTGCGAGTGC-3' and 5'-ATGGGGCCGC TCGAGTCACT GACAGCAGCT AGTG-3'. A glutathione S-transferase (GST)-MT fusion protein was formed in pGEX-4T-1 vector (Amersham, UK) by ligating the MT PCR product to the vector through the two restriction sites. The recombinant vector was transfected into competent BL21-Gold (3D) bacterial strain (Stratagene, San Diego, USA). Selected BL21 colony was grown overnight at 37°C with shaking (~250 rpm). Isopropyl β -D-thiogalactopyranoside (IPTG) and Zn⁺⁺ were added at mid-exponential bacterial growth phase (OD₆₀₀ ~ 0.6) to final concentrations of 0.1 mM and 300 μ M, respectively. Induced cells were grown for additional four hours, harvested by centrifugation, and washed in PBS (8.1 mM Na₂HPO₄, 1.47 mM KH₂PO₄, 137 mM NaCl, and 2.7 mM KCl; pH 7.3) containing 5 mM dithiothreitol (DTT). Bacterial cells were lysed by their resuspension in PBS (4 ml/100 ml culture) containing 5 mM DTT and supplemented with protease inhibitors cocktail and 1 mg ml⁻¹ lysozyme, mildly rocked for 30 min at 4°C. 2.5 volumes of 0.2% triton X-100 (in PBS) was injected forcibly into the viscous cell lysate concurrent with 1 μ g ml⁻¹ DNase and the lysate was rocked for additional 10-20 min. The lysate was centrifuged at 10,000 g for 30 min, and the supernatant was recovered and supplemented with 1 mM DTT. The GST-MT fusion protein was purified by batch affinity chromatography with glutathione-Sepharose-4B beads (Pharmacia, Uppsala, Sweden) at a volume ratio of 1/10 (matrix/sample). The mixture was incubated with gentle agitation for 30-60 min at room temperature, and after washing (\times 3) in PBS, the resin was packed in a column and the MT-GST was eluted with glutation/Tris buffer (10 mM reduced glutathione in 50 mM Tris-HCl; pH 8). MT-GST containing fractions were 10-fold concentrated using Centriprep concentrators (Amicon, Billerica, USA; cutoff of 3 kDa) and the MT was cleaved from the GST in thrombine-PBS solution (10 units

thrombine mg⁻¹ fusion protein, Pharmacia) for 16h at 22°C. To that end, the solution was heated for 30 min at 75-80°C, followed by centrifugation at 20,800 g for 20 min.

L. mormyrus cytosolic fraction was separated from hepatic homogenates in PBS by ultracentrifugation according to Tom et al. (2002) and the resulting supernatant was heated to 70°C for 20 min. The denatured proteins were precipitated at 20,800 g for 20 min and the resulting supernatant was concentrated as required for visualization of the MT by Western blot.

An anti-MT antibody was produced in rabbits using the recombinant protein as antigen but did not immunoreact with the native protein in the ELISA solution, therefore, replaced by a much less sensitive commercial polyclonal anti-Cod IgG (Abcam, Cambridge, UK; #ab36882).

Mass spectrometry sequencing of the recombinant MT

Recombinant MT (1 mg) in PBS was freeze-dried and dissolved in 20 µl of 8 M urea and 0.4 M NH₄HCO₃ buffer. Subsequently, 20 µl of EDTA and 5 µl of 45 mM DTT were added and the sample was incubated at 50°C for 15 min. After cooling down to room temperature, 5 µl of 100 mM of iodoacetamide was added and the solution was incubated for 30 min at room temperature in the dark. The reduced and alkylated protein was digested with 2% trypsin (Promega, Madison, WI, USA,) for 24 h and the peptide mixture was separated using high performance liquid chromatography on AKTAexplorer™ system (GE Healthcare, UK). The fractions were analyzed by Matrix Assisted Laser Desorption-Time of Flight-tandem mass spectrometry (MADLI-TOF MS/MS) using the 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA).

Synthesis of recombinant beta-actin

The coding region of *L. mormyrus* beta-actin cDNA was reverse transcribed. The resulting cDNA was modified to contain the PstI and EcoRI restriction sites, as well as a 5×histidine tag at its carboxy terminus. The modification was carried out by PCR (95°C–2 min; 35 cycles of [95°C–1 min, 55°C–1 min, 72°C–3 min]; 72°C–2 min) using the primer pair 5'-ATGGAAGATG

AAATCGCCGC AC-3' (forward/PstI) and 5'-CTAATGGTGATGG ***TGATGGAAGC*** ATTTGCGGTG GA CG-3' (reverse/EcoRI; 5×histidine tag in bold italics). The primers were designed according to the beta-actin sequence of the related sparid fish *Sparus aurata* (GenBank Accession no. X89920). Beta-actin was produced in Sf9 insect cells according to the instructions of the production kit manufacturer (Pharming, San Diego, CA, USA) excluding the Sf9 cell lysis procedure. Briefly, the modified cDNA was cloned into the pVL1392 baculovirus transfer vector and then co-transfected with the Baculogold viral DNA into Sf9 insect cells, maintained in monolayer culture. Cells were harvested 72h post-infection by low speed centrifugation at 100 g for 5 min, and lysed for 30 min at 4°C in insect cell lysis buffer (0.5 ml/100 mm plate) composed of 50 mM sodium phosphate buffer (pH 7.4), 450 mM NaCl, 1% Triton X-100 and 10% glycerol. The lysis buffer was supplemented prior to use with 5 mM beta-Mercaptoethanol and protease inhibitors cocktail. Cellular debris was removed from the lysate by centrifugation at 20,800 g for 20 min. Recombinant Beta-actin was purified from the supernatant by affinity chromatography on a Ni—NTA column (Qiagen, Hilden, Germany) which was eluted from the column by a buffer which contained 200 mM imidazole. Both the recombinant and the native fish beta-actin reacted specifically with a commercial rabbit anti-actin IgG (Santa Cruz, Santa Cruz, CA, USA; #sc-1616r ,200 µg ml⁻¹), immunoreacting with conserved actin C-terminus.

Fish sampling, maintenance and experimental manipulations

Fish were sampled alive in the winter from their natural habitats at the Mediterranean coast of Israel by gillnetting. A total of 44 striped sea bream individuals, sampled at the same date and site were used for the induction experiment. Another 112 fish were sampled at two coastal sites in the winters of 2004-2005 and were sacrificed three hours after sampling. The sediment and the biota of the Haifa Bay site are located adjacent to an industrial region and the Haifa Harbor and contain higher levels of transition metals than other sites along the Israeli coast of the Mediterranean. The second site consists of two locations 10-25 km south of Haifa bay that were considered clean sites

(Herut et al., 1993; 2006). Fish were maintained at the laboratory, before and during the experiment, in 0.7 m³ tanks supplied with flow-through Mediterranean coastal seawater at ambient winter seawater temperature of 16-21°C, fed by commercial fish food pellets (Cat. # 1369, Matmor central feed mill, Ashqelon, Israel).

Cadmium-containing food was prepared by grinding the food pellets and their repackaging as a mixture of 63% food powder, 35% CdCl₂ aqueous solution (200 or 20 µg CdCl₂ ml⁻¹) and 2.5% gelatin as solidifier, dried overnight at 45-50°C. Cadmium was applied also through 50 µl intra peritoneal injections of CdCl₂ solution in saline (2 mg cadmium ml⁻¹).

Fish were decapitated at the termination of each experiment or immediately after sampling, followed by an immediate dissection of liver and gonads. Livers were divided into smaller aliquots, snap frozen in liquid nitrogen and kept at -80°C. Gonads were fixated in 4% formaldehyde solution. Fish reproductive activity was determined by microscopic observation of gonads smeared between two slides, measuring the average ova diameter of 20 oocytes. Being proteandrous hemaphrodite, *L. mormyrus* ovotestes contained always at least primordial oogonia.

MT induction experiment

Table 1 provides detailed description of each experimental group of fish, including treatment, total number of fish, average fish weight, average oocyte diameter, and description of the treatment. Protein ELISA evaluations were carried out on protein pools, as the futile examinations of the in-house produced antibody (see above) left us with too small protein samples. The number of evaluated protein samples is also detailed in table 1.

MT, beta-actin and cytochrome P4501A competitive ELISAs

Fish hepatic homogenates, 500 µl/60-80 mg tissue, were prepared in PBS, supplemented prior to use, with 5 mM beta-Mercaptoethanol and protease inhibitors cocktail. Debris was eliminated by centrifugation at 10,000 g for 15 min, saving the supernatant. Purified recombinant cytochrome

P4501A, MT and beta-actin were used as standards and their concentrations were determined by the modified Lowry method (DC protein assay kit, BioRad, Hercules, CA, USA). The competitive ELISAs were carried out according to Tom et al. (2002) which described the ELISA of cytochrome P4501A. Triton X-100 application at the competition stage was implemented only for the ELISA of cytochrome P4501A.

A matrix of several concentrations of coating MT (100, 300, 500 nM) and antibody dilutions (1:2,000; 1:3,500; 1:5,000) were applied to optimize the ELISA. Similarly, coating beta-actin concentrations of 10, 50 nM and antibody dilutions of 1:10,000 and 1:20,000 were applied to optimize the corresponding ELISA. The optimization procedure was aimed at achieving wide dynamic range. The detailed optimization procedure is described in Tom et al. (2002).

Serial dilutions of the standard protein and the samples were performed for each ELISA. Both the standard and the sample log-linear curves were used to identify the linear range of the immunochemical competition reaction, which was used for the analysis. Similarity of the curves' slopes between the sample-containing and the standard-containing ELISA wells signified similar immunochemical competition kinetics, an accuracy determining factor.

Evaluation of hepatic MT transcript levels

Total hepatic RNA was extracted using the EZ II kit (Biological Industries, Beit Haemek, Israel) or by the RNeasy purification kit (Qiagen), according to the manufacturers' instructions. The quality of all produced RNAs was evaluated by electrophoresis on a 1% agarose-formaldehyde gel and their concentration was evaluated by spectrophotometry.

MT and beta-actin transcript levels were evaluated by real time PCR according to Tom et al (2004) with a modification. The RNA templates for the reverse transcription reactions prior to the real time PCR were brought to a concentration of $0.5 \text{ ng } \mu\text{l}^{-1}$ and a volume of $2 \mu\text{l}$ was applied into each reaction tube. Two μl of the resulted reverse transcription solution was added to each real time

PCR reaction. The increased volumes in comparison to Tom et al. (2004) and the equalized RNA levels were aimed at decreasing the variation among replicates.

Measurements of cadmium levels in fish liver

Cadmium concentrations were determined on approximately 0.1 g of dry liver tissue that was digested for 4 h at 140°C with concentrated nitric acid (65 wt %) in Uniseal, teflon-lined, high pressure decomposition vessels according to Herut and Kress (1997). The digests were diluted to exactly 15 ml in clean plastic tubes with double distilled water. Measurements were performed by Varian SpectrAA220 flame atomic absorption and SpectrAA880 graphite furnace. The accuracy and precision of the methods were evaluated on the basis of analysis of several international standard reference materials (DOLT3, DORM-2, IAEA 407). All elements gave results within 5% of the certified values.

Results

Production of recombinant MT

The production and the purification procedure of the recombinant MT are demonstrated in Fig. 1, as well as the immunochemical affinity of both the anti-recombinant *L. mormyrus* MT and the commercial anti-cod MT to the recombinant protein and to hepatic extract of *L. mormyrus*. The isolated MT was identified as the major end product resulting from the purification procedure. The identity of the purified protein as MT was further demonstrated by mass spectrometry. Full sequences of 4 peptides (GSPEFMDPCECAK, TGTCNCGGSCSCTNCSTCK, KSCCSCPAGCSK, SCCSCPAGCSKASGCVCK) were elucidated, with the third and the fourth peptides partially overlapping. An overall identity to 84% of the recombinant protein sequence was obtained. Peptides corresponding to the last 10 amino acids (GKTCDTSCCQ) were not recovered being fragmented into small peptides not detectable by the MALDI TOF-MS even

when using an incomplete digestion approach. The first five amino acids of the first peptide (GSPEF) belong to the thrombin restriction site introduced by the pGEX-4T-1 vector.

Although protected from oxidation throughout the purification procedure, the recombinant MT revealed the typical ladder-like profile on SDS-PAGE, unlike the dimer conformation of the native MT. The antibody that was produced by us in rabbits using the recombinant protein as antigen was more sensitive than the commercial anti-cod MT when tested on solid surface. However, it had no affinity to the native MT in solution.

Production of recombinant beta-actin

The pure recombinant beta-actin revealed the appropriate actin molecular weight. Its identity was further demonstrated by its positive and specific immunochemical affinity to the commercial anti-actin which reacted also with the fish native protein. The resulting products of the various stages of the purification procedure and their immunochemical affinity to the antibody are presented in Fig. 2.

MT and beta-actin competitive ELISAs

Typical ELISA results of the two proteins, including the final optimized standard protein concentrations are presented in Fig. 3a (MT) and Fig. 3b (beta-actin). The optimal antibody dilutions were 1:5,000 (MT) and 1:20,000 (beta-actin). Optimal coated MT concentration was 300 nM ml⁻¹ and the optimal coated beta-actin concentration was 10 nM ml⁻¹. Only the linear part of the ELISA reaction was used for the analysis of protein levels. It has to be emphasized that plate to plate variations in the binding capacity of the walls of the ELISA wells to the coated antigen may change the slope and the linear range of the competition reaction curve and the linear range has to be identified for each ELISA plate.

MT induction experiment

The results of the MT induction experiment are presented in Fig. 4. Levels of hepatic cadmium were normalized to liver wet weight, whereas MT transcript and protein were normalized to the respective levels of beta-actin transcript and protein.

Sampling of fish from natural habitats

The hepatic levels of MT and cytochrome P4501A, as well as cytochrome P4501A protein level in the sampled fish were evaluated. The results are detailed in Table 2. A statistically significant difference (t-test; $p < 0.05$) was found between the two sampled sites for all the measured biomarkers, always higher in Haifa Bay. The gonads of all sampled fish were in reproduction arrest, revealing average sample oocyte diameters of: 83 ± 30 , 69 ± 42 , 53 ± 26 and 61 ± 32 μm , all below the size of reproductive females (Funkenstein et al., 2004).

Discussion

The cadmium exposure experiment was aimed at creating several exposure intensities for testing the ELISAs. However, limited discussion of cadmium transportation to the liver and its sequestration by MT can be done in view of the results. Hepatic bio-accumulated cadmium levels corresponded to the applied cadmium levels for both types of treatment. The higher and lower ingested cadmium doses were 17.7-fold apart and the corresponding cadmium levels differed by 15.1-fold. The injected doses and the corresponding hepatic cadmium levels were both 4-fold apart. Therefore, it can be postulated that cadmium rapidly reaches the liver, and during the experimental time scale was accumulated there. However, absorption of cadmium in the liver was much more efficient through the pharmacological injection route (17-18%) than through the physiological ingestion route (0.32-0.44%). Similar continuous cadmium accumulation was found in other fish cadmium exposure studies during a time frame of up to four months of exposure (Berntssen et al., 2001; Chowdhury et al., 2005). The accumulated cadmium amounts in this study were very high due to the extreme exposure, indicating very high capacity of the liver for cadmium. Cadmium is

accumulated in the liver and its further transportation to other organs is limited (Wicklund et al., 1988). It seems that a chronic exposure which may lead to steady state cadmium hepatic levels requires long-term exposure to high cadmium levels and most exposure experiments, including ours, did not reach this equilibrium.

The results may be interpreted in view of Campbell et al. (2005) who studied cadmium distribution in perch liver under several natural long-term, chronic exposure regimes to waterborne cadmium. They showed that fish hepatic cellular components, fractionated by differential centrifugation, sequestered cadmium in constant ratios. Cadmium was trapped by both cadmium-sensitive and resistant cellular components and the bio-accumulated amounts were in correlation with the exposure intensity. However, most of the cadmium (~60%) was located in the heat stable cytosolic component, probably trapped by MT, protecting the liver from cadmium toxicity. In the present study, the two cadmium-fed doses caused a 5-6-fold increase of both the MT transcript and protein levels. The cadmium application through feeding can be considered closer to equilibrium-state as determined by Campbell et al. (2005) than the injected cadmium, as it was continuously provided to the fish over a two months period. The increased cadmium levels were sequestered by the increasing MT levels. One MT molecule can sequester 6-7 cadmium molecules (Hamer, 1986). Therefore, a 5-6 fold MT increase explained the trapping of 15-fold increased cadmium levels. In contrast, injected cadmium represent a pharmacological non-equilibrium state. Cadmium quickly entered the liver but the transcript and protein levels were still changing. Naturally, elevated MT transcript level, preceded the increase in protein levels, and the different durations of cadmium exposure between the two applied doses, 8 and 12 days for the low and high doses, respectively (Table 1) may explain the disproportional increase of the MT protein upon the high injection dose. This extremely high MT level demonstrates high protection capacity of the liver from cadmium toxicity.

MT transcript level in the striped sea bream liver is used by us as an environmental biomarker, responding to the presence of environmental transition metals and other MT-affecting parameters.

However, exposure experiments to cadmium revealed MT transcript induction only upon exposure to relatively high levels of cadmium, not usually existing in the coastal environment of the Mediterranean. In this study, significantly induced MT transcript was related to a minimum of 7.4 μg cadmium/g wet liver weight, 10-20 fold higher than elucidated levels of cadmium and other MT inducing metals in the polluted Haifa Bay. Similar insensitivity was revealed by Bourdineaud et al. (2006) applying environmentally-realistic low waterborne cadmium to zebrafish, revealing no MT induction and Williams et al. (2006) who applied low cadmium levels to flounders with only faint and transient induction. A cadmium level of 0.49 μg cadmium/g wet liver weight, which was elucidated in one of the treatment groups, is much closer to real environmental levels, and it revealed no significant inducible MT transcript levels. However, the comparison of MT transcript levels between two sampling sites along the Israeli coast, revealed consistent differences in the levels of several biomarkers, including MT transcript, between polluted and clean sites over two years sampling period. Therefore, it can be preliminarily concluded that the chronic environmental influence over extended periods of time, composed of multiple effectors, evoked significant MT and also cytochrome P4501A induction, the latter emphasizes the multi-factorial nature of the pollution in the examined habitat. The partially undefined natural pollutant mixture is composed of cadmium and other transition metals (Herut et al., 1993, 2006) and also other unknown components. Acute intensive cadmium exposures are interesting as they test the extremes of endurance and protection capacity. However, they do not mimic realistic environmental conditions which involve long-term exposures to low levels of multi-pollutant mixtures.

Recombinant MT and beta-actin standard proteins of the striped sea bream and suitable anti-MT and anti-actin antibodies were integrated into two competitive ELISAs to fit the striped sea bream MT protein evaluation and to improve its comparability. The beta-actin ELISA can also be used as a normalizing agent for the previously developed striped sea bream competitive ELISAs of cytochrome P4501A (Tom et al., 2002) and vitellogenin (methods are reviewed by Tom and Auslander, 2005). The striped sea bream protein and transcript biomarkers, described here and in

Tom et al. (2003, 2004) and in Funkenstein et al. (2004) contain the components required for their implementation as comparable diagnostic environmental biomarker assays as depicted in the introduction above. However, establishment of these ELISAs as routine environmental assays requires further attention to two aspects: accurate and uniform protocol for standard protein evaluations, and improvement of the normalization procedure. The modified Lowry method used here for protein standard evaluation is not sufficiently accurate and the utilized protein standards used with the Lowry method (bovine serum albumin, gamma-globulin, etc.) and their sources are not widely accepted and are subject to the decision of each laboratory.

Proper normalization agent should be a protein which is found in constant amounts per cell or tissue mass at all biological situations and also located in the same homogenized fraction as the target protein, and therefore exposed to the same homogenization procedure with similar extraction efficiency. It is difficult to find one universal protein to serve as a normalizing agent. Therefore, several agents have to be examined for every biomarker assay, selecting those which show better correlation with each other, statistically eliminating non-adequate ones. One protein, beta-actin, was provided by this study. It is a widely used normalizing agent and being present in the supernatant of the S9 fraction, it is adequate as MT, cytochrome P4501A and vitellogenin normalizing agent. The above discussion of measurement of standards and proper normalization is valid also to transcript evaluations.

Conclusions

Competitive ELISA for the measurement of MT levels was modified to be used in the sentinel fish *L. mormyrus*, alongside with a similar procedure for evaluating beta-actin levels to be used as a normalizing agent of MT and other evaluated biomarker proteins. Almost all the components of these ELISA procedures were described earlier in fish, excluding the production of recombinant fish beta-actin. However, their assembly into one environmentally-appropriate protocol may be considered the major contribution of this study. Two aspects of this biomarker evaluation system

still require improvement: 1) accurate evaluation of the standards and 2) provision of additional normalizing agents for the selection of the best one for this assay. Both the transcript and the protein can serve as environmental biomarkers. It was demonstrated that although intensive controlled exposure to cadmium is required to induce MT transcript levels, the levels of MT in feral fish were significantly and repeatedly different between a polluted and clean natural habitats, although the accumulated level of metals in the sediment was lower than the level of the experimentally applied cadmium.

Acknowledgement

This study was supported by EU GENIPOL project (EVK-2001-00057). Special appreciation is extended to Ms. Maria Segal for her excellent care of the fish.

References

- Alvarado, N.E., Quesada, I., Hylland, K., Marigomez, I., Soto, M., 2006. Quantitative changes in metallothionein expression in target cell-types in the gills of turbot (*Scophthalmus maximus*) exposed to Cd, Cu, Zn and after a depuration treatment. *Aquatic Toxicology* 77, 64-77.
- Atli, G., Canli M., 2003. Natural Occurrence of Metallothionein-Like Proteins in the Liver of Fish *Oreochromis niloticus* and Effects of Cadmium, Lead, Copper, Zinc, and Iron Exposures on Their Profiles. *Bulletin of Environmental Contamination and Toxicology* 70, 619-627.
- Bauchot, M.L., Hureau, J.C., 1986. Sparidae. In: Whitehead, P.J.P., Bauchot, M.L., Hureau, J.C., Nielsen, J., Tortonese, E., (Eds.), *Fishes of the north-eastern Atlantic and the Mediterranean*. UNESCO, U.K., Vol. II: pp. 883-897.
- Berntssen, M.H.G., Aspholm, O.O., Hylland, K., Bonga, S.E.W., Lundebye, A.K., 2001. Tissue metallothionein, apoptosis and cell proliferation responses in Atlantic salmon (*Salmo salar* L.) parr fed elevated dietary cadmium. *Comparative Biochemistry and Physiology, C-Toxicology and Pharmacology* 128, 299-310.

- Besseau L., 1990. Etude Histo-Cytologique de la structure sexuelle d'une population de *Lithognathus mormyrus* (L.) (Teleosteen, Sparide). Rapport et Procès-Verbaux des Réunion Commission Internationale pour L'exploration Scientifique de la Mer Méditerranée 32, 262-266.
- Bourdineaud, J.P., Baudrimont, M., Gonzalez, P., Moreau, J.L., 2006. Challenging the model for induction of MT gene expression. *Biochimie* 88, 1787-1792.
- Buxton, C.D., Smale, M.J., Wallace, J.H., Cockcroft, V.G., 1984. Inshore small-mesh trawling survey of the Cape south coast. Part 4. Contributions to the biology of some Teleostei and Chondrichthyes. *South African Journal of Zoology* 19, 182-189.
- Buxton, C.D., Garratt P.A., 1990. Alternative reproduction styles in seabreams (Pisces, Sparidae). *Environmental Biology of Fishes* 28, 113-124.
- Campbell, P.G.C., Giguere, A., Bonneris, E., Hare, L., 2005. Cadmium-handling strategies in two chronically exposed indigenous freshwater organisms - the yellow perch (*Perca flavescens*) and the floater mollusc (*Pyganodon grandis*) *Aquatic Toxicology* 72, 83- 97.
- Chan, K.M., Davidson, W., Hew, C., Fletcher, G., 1989. Molecular cloning of metallothionein cDNA and analysis of metallothionein gene expression in winter flounder tissues. *Canadian Journal of Zoology* 67, 2520-2527.
- Chowdhury, M.J., Baldisserotto, B., Wood, C.M., 2005. Tissue-specific cadmium and MT levels in rainbow trout chronically acclimated to waterborne or dietary cadmium. *Archives of Environmental Contamination and Toxicology* 48, 381-390.
- Coyle, P., Philcox, J.C., Carey, L.C., Rofe, A.M., 2002. Metallothionein: the multipurpose protein. *Cellular and Molecular Life Sciences* 59, 627-647.
- De Boeck, G., Ngo, T.T.H., Van Campenhout, K., Blust, R., 2003. Differential metallothionein induction patterns in three freshwater fish during sublethal copper exposure. *Aquatic Toxicology* 65, 413-424.
- Eroglu, K., Atli, G., Canli, M., 2005. Effects of metal (Cd, Cu, Zn) interactions on the profiles of

- metallothionein-like proteins in the Nile fish *Oreochromis niloticus*. *Bulletin of Environmental Contamination and Toxicology* 75, 390-399.
- Frogia, C., 1977. Feeding of *Lithognathus mormyrus* (L.) in the Central Adriatic Sea (Pisces, Sparidae). *Rapport et Procès-Verbaux des Réunion Commission Internationale pour L'exploration Scientifique de la Mer Méditerranée* 24, 95-97.
- Funkenstein, B., Dyman, A., Levavi-Sivan, B., Tom, M., 2004. Application of real time PCR for quantitative determination of hepatic vitellogenin transcript levels in the striped seabream, *Lithognathus mormyrus*. *Marine Environmental Research* 58, 659-663.
- George, S.G., Olsson, P-A., 1994. Metallothioneins as indicators of trace metal pollution. In: *Biomonitoring of coastal waters and estuaries*. Kees, J., Kramer, M., (Eds.).
- George, S.G., Todd, K., Wright, J., 1996. Regulation of metallothionein in teleosts: Induction of metallothionein mRNA and protein by cadmium in hepatic and extrahepatic tissues of a marine flatfish, the turbot (*Scophthalmus maximus*). *Comparative Biochemistry and Physiology* 113, 109-115.
- Hamer, D.H., 1986. Metallothionein. *Annual Review in Biochemistry* 55, 913-951.
- Handy, R.D., Galloway, T.S., Depledge, M.H., 2003. A proposal for the use of biomarkers for the assessment of chronic pollution and in regulatory toxicology. *Ecotoxicology* 12, 331-343.
- Hansen, B.H., Romma, S., Garmo, O.A., Olsvik, P.A., Andersen, R.A., 2006. Antioxidative stress proteins and their gene expression in brown trout (*Salmo trutta*) from three rivers with different heavy metal levels. *Comparative Biochemistry and Physiology C-Toxicology and Pharmacology* 143, 263-274.
- Herut, B., Hornung, H., Krom, M.D., Kress, N., Cohen Y., 1993. Trace metals in shallow sediments from the Mediterranean coastal region of Israel. *Marine Pollution Bulletin* 26, 675-682.
- Herut, B., Kress, N., 1997. Particulate Metals Contamination in the Kishon River Estuary, Israel. *Marine Pollution Bulletin* 34, 706-711.

- Herut, B., Cohen, Y., Shefer, E., Gordon, N., Galil, B., Tibor, G., Tom M., 2006. Environmental quality of Israel's Mediterranean coastal waters in 2005, IOLR Report H25/2006. Executive summary in English.
- Hylland, K., 1999. Biological effects of contaminants: quantification of metallothionein in fish. ICES Techniques in Marine Environmental Sciences No. 26, 18 pp.
- Joel, P.B., Fagnant, P.M., Trybus, K.M. 2004. Expression of a nonpolymerizable actin mutant in Sf9 cells. *Biochemistry* 43, 11554-11559.
- Kagi, J.H.R., 1991. Overview of metallothionein. *Methods in Enzymology* 205, 613-626.
- Kraljevic, M., Dulcic, J., Pallaoro, A., Cetinic, P., Jug Dujakovic, J., 1995. Sexual maturation, age and growth of striped sea bream, *Lithognathus mormyrus* L., on the eastern coast of the Adriatic Sea. *Journal of Applied Ichthyology* 11, 1-8.
- Lasiak, T.A., 1984. Aspects of the biology of three benthic-feeding teleosts from Kingmins Beach, Algoa Bay. *South African Journal of Zoology*, 1951-1956.
- Long, A.M., Wang, W.X., 2005. Metallothionein induction and bioaccumulation kinetics of Cd and Ag in the marine fish *Terapon jarbua* challenged with dietary or waterborne Ag and Cu. *Marine Ecology-Progress Series* 291, 215-226.
- Micale, V., Perdichizzi, F., 1994. Further studies on the sexuality of the hermaphroditic teleost. *Journal of Fish Biology* 45, 661-670.
- Olsvik, P.A., Hindar, K., Zachariassen, K.E., Andersen, R.A., 2001. Brown trout (*Salmo trutta*) metallothioneins as biomarkers for metal exposure in two Norwegian rivers. *Biomarkers* 6, 274-288.
- Sambrook, J., Russell, D.W., 2001. *Molecular cloning, a laboratory manual*. 3rd edition, Cold Springs Harbor: Cold Springs Harbor Laboratory Press.
- Suau, P., 1970. Contribucion al estudio de la biologia de *Lithognathus (Pagellus) Mormyrus* L. (Peces esparidos). *Investigacion Pesquera* 34, 237-265.

- Tom, M., Jakubov, E., Rinkevich, B., Herut, B., 1999. Monitoring of hepatic metallothionein mRNA levels in the fish *Lithognathus mormyrus* - evaluation of transition metal pollution in a Mediterranean coast. *Marine Pollution Bulletin* 38, 503-508.
- Tom, M., Myers, C.R., Waterman, M.R., 2002. Competitive ELISA utilizing membrane-free recombinant fish CYP1A as standard for evaluation of its microsomal homologue molar concentrations. *Aquatic Toxicology* 59, 101-114.
- Tom, M., Shmul, M., Shefer, E., Chen, N., Slor, H., Rinkevich, B., Herut, B., 2003. Quantitative evaluation of hepatic cytochrome P4501A transcript, protein and catalytic activity in the striped sea bream, *Lithognathus mormyrus*. *Environmental Toxicology and Chemistry* 22, 2088–2092.
- Tom, M., Chen, N., Segev, M., Herut, B., Rinkevich, B., 2004. Quantifying fish metallothionein transcript by real time PCR for its utilisation as an environmental biomarker. *Marine Pollution Bulletin* 48, 705-710.
- Tom, M., Auslander, M., 2005. Transcript and protein environmental biomarkers in fish – a review. *Chemosphere* 59, 155–162.
- Van der Oost, R., Beyer, J., and Vermeulen., N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental Toxicology and Pharmacology* 13, 57-149.
- Vergani, L., Grattarola, M., Dondero, F., Viarengo, A., 2003. Expression, purification, and characterization of MT-A from rainbow trout. *Protein Expression and Purification* 27, 338-345.
- Wicklund, A., Runn, P., Norrgren, L., 1988. Cadmium and zinc interactions in fish: effects of zinc on the uptake, organ distribution, and elimination of ¹⁰⁹Cd in the zebrafish *Brachydanio rerio*. *Archives of Environmental Contamination and Toxicology* 17, 345-354.
- Williams, T.D., Diab, A.M., George, S.G., Godfrey, R.E., Sabine, V., Conesa, A., Minchin, S.D., Watts, P.C., Chipman, J.K., 2006. Development of the GENIPOL European flounder (*Platichthys flesus*) microarray and determination of temporal transcriptional responses to cadmium at low dose. *Environmental Science and Technology* 40, 6479-6488.

- Woo, S., Yum, S., Jung, L.H., Shim, W.J., Lee, C.H., Lee, T.K., 2006. Heavy metal-induced differential gene expression of metallothionein in Javanese medaka, *Oryzias javanicus*. *Marine Biotechnology* 8, 654-662.
- Wu, S.M., Weng, C.F., Yu, M.J., Lin, C.C., Chen, S.T., Hwang, J.C., Hwang, P.P., 1999. Cadmium-Inducible Metallothionein in Tilapia (*Oreochromis mossambicus*). *Bulletin of Environmental Contamination and Toxicology* 62, 758-768.
- Wu, S.M., Weng, C.F., Hwang, J.C., Huang, C.J., Hwang, P.P., 2000. Metallothionein Induction in Early Larval Stages of Tilapia (*Oreochromis mossambicus*). *Physiological and Biochemical Zoology* 73, 531-537.
- Yang, F., Zhou, M., He, Z.M., Liu, X.R., Sun, L., Sun, Y., Chen, Z.C., 2007. High-yield expression in *Escherichia coli* of soluble human MT2A with native functions. *Protein Expression and Purification* 53, 186-194.

Table 1: Characterization of the cadmium exposure experiment. Each experimental group is designated and its total number of fish, total number of pooled protein samples, average fish weight, average oocyte diameter and detailed treatment description is provided. IP – intra-peritoneal.

Group	No. of fish	Number of protein samples	Fish weight [gr]	Average oocyte diameter [μ m]	Treatment description
C	8	3	38.8 \pm 5.4	67 \pm 26	31 aliquots of food pellets containing no cadmium, each aliquot of 1% body weight, four times a week, sacrificed 3 days after last feeding.
LF	10	3	39.7 \pm 4.2	74 \pm 57	31 aliquots of food pellets containing 3.23 μ g cadmium/gr food, each aliquot of 1% body weight, four times a week, sacrificed 3 days after last feeding.
HF	10	3	36.2 \pm 5.1	61 \pm 36	31 aliquots of food pellets containing 57.25 μ g cadmium/gr food, each aliquot of 1% body weight, four times a week, sacrificed 3 days after last feeding.
Ix1	9	2	40.4 \pm 6.2	70 \pm 34	One IP injection of 50 μ l Cd in saline (2 mg/ml), sacrifice after 8 days.
Ix4	7	1	40.3 \pm 4.6	63 \pm 30	Four IP injections of 50 μ l Cd in saline (2 mg/ml) at 3 days intervals, sacrificed 4 days after fourth injection

Table 2: Bio-monitoring of the Israel Mediterranean coast. Results are presented as average \pm standard deviation. Fish sample size is presented within parentheses.

Biomarker	Haifa Bay - Polluted site	10-25 km south to Haifa Bay - Relatively clean sites
Transcript (2004) - Biomarker / Actin [atomol/atomol]		
Metallothionein	0.43 \pm 0.40 (20)	0.24 \pm 0.13 (8)
Cytochrome P4501A	2.07 \pm 1.48 (20)	0.53 \pm 0.25 (8)
Transcript (2005) – Biomarker / Actin [atomol/atomol]		
Metallothionein	0.79 \pm 0.48 (20)	0.31 \pm 0.47 (20)
Cytochrome P4501A	3.84 \pm 3.86 (8)	0.54 \pm 0.36 (8)
Protein (2004) [nmol mg⁻¹ total protein]		
Cytochrome P4501A	27.9 \pm 19.9 (20)	5.3 \pm 4.8 (8)

Figure captions

Fig. 1: *L. mormyrus* MT was expressed in *E. coli* as MT-GST fusion protein, using the pGEX-4T-1 vector. Lane 1 - Bacterial lysate. Lane 2 – Purified MT-GST fusion protein, isolated on glutathione-Sepharose-4B affinity column. Lane 3 - Enzymatic cleavage of the fusion protein by thrombin, inserted between the GST and the MT. Lanes 4 and 5 – Two doses of the purified MT. Almost all the GST was precipitated from the solution by centrifugation after its heating to 70°C. Lanes 6, 7 – Immunoblot of the anti-*Lithognathus* recombinant MT against fish liver lysate and recombinant MT, respectively. Lanes 8, 9 – Immunoblot of the commercial anti-cod-MT against fish liver lysate and recombinant MT, respectively. rMT – recombinant metallothionein, X- residual GST.

Fig. 2: Recombinant 5×his tagged beta-actin was produced in Sf9 insect cells. Lane 1 - fish liver lysate. Lane 2 – Sf9 cell lysate. Lane 3 - Purified beta-actin, isolated from Sf9 cell lysate on Ni-NTA 5×His tag affinity column. A commercial anti human-actin antibody demonstrated specific affinity with the fish beta-actin.

Fig. 3: Examples of the results of MT and beta-actin ELISAs. Y axis – absorbance at 405 nm in arbitrary units. A and C are calibration curves and B and D are sample dilution curves. MT- metallothionein. Only the linear part of each curve was included in the figure. Liver protein homogenates were 50-fold diluted for the actin ELISA to meet optimal assay conditions.

Fig. 4: Normalized hepatic levels of cadmium as well as MT transcript and protein in the various experimental treatments (see table 1 for treatments' designations). Cadmium and MT transcript levels are averages of measurements in individual fish, whereas pools of fish were used for the evaluation of protein levels. * - statistically significant differences from the control (t-tests, $p < 0.05$).

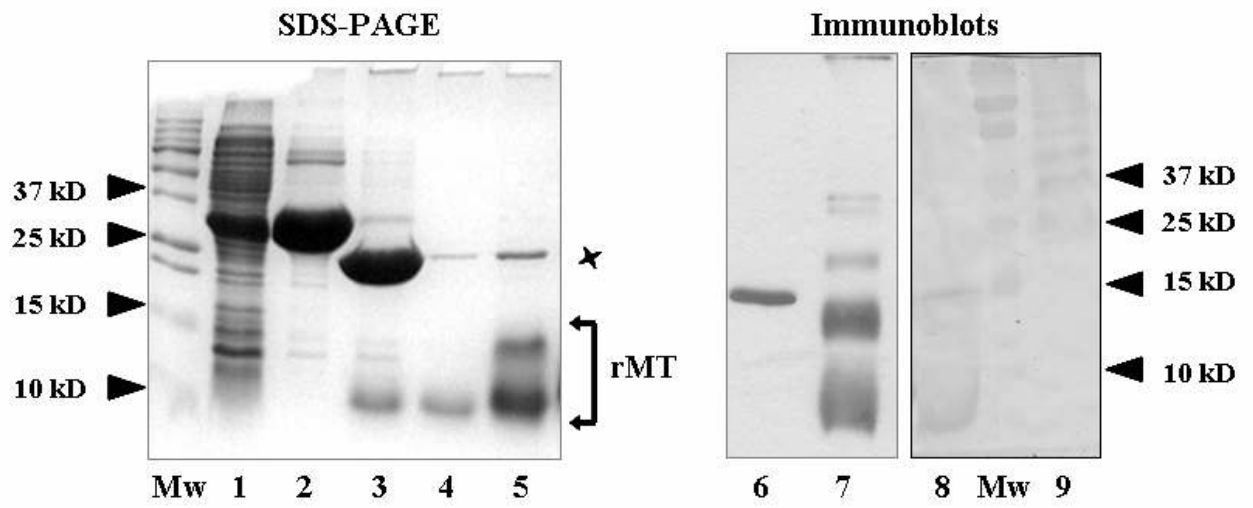
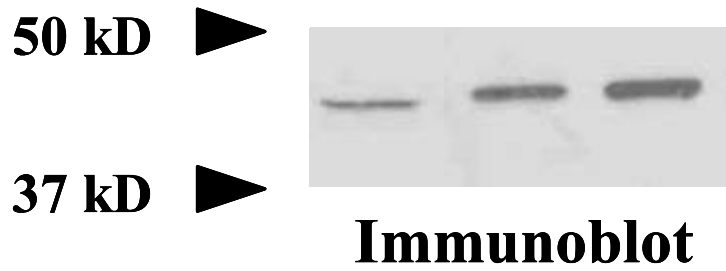
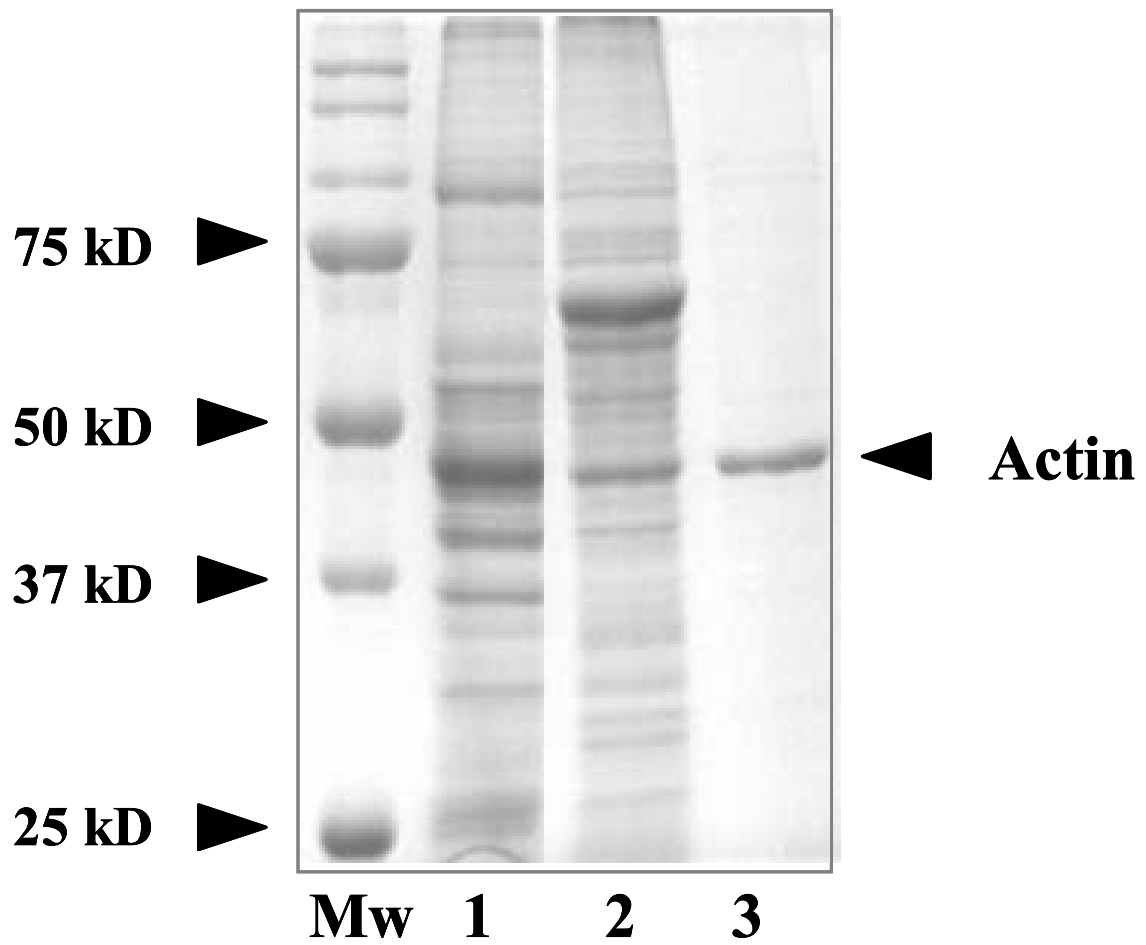


Fig. 1

SDS-PAGE**Fig. 2**

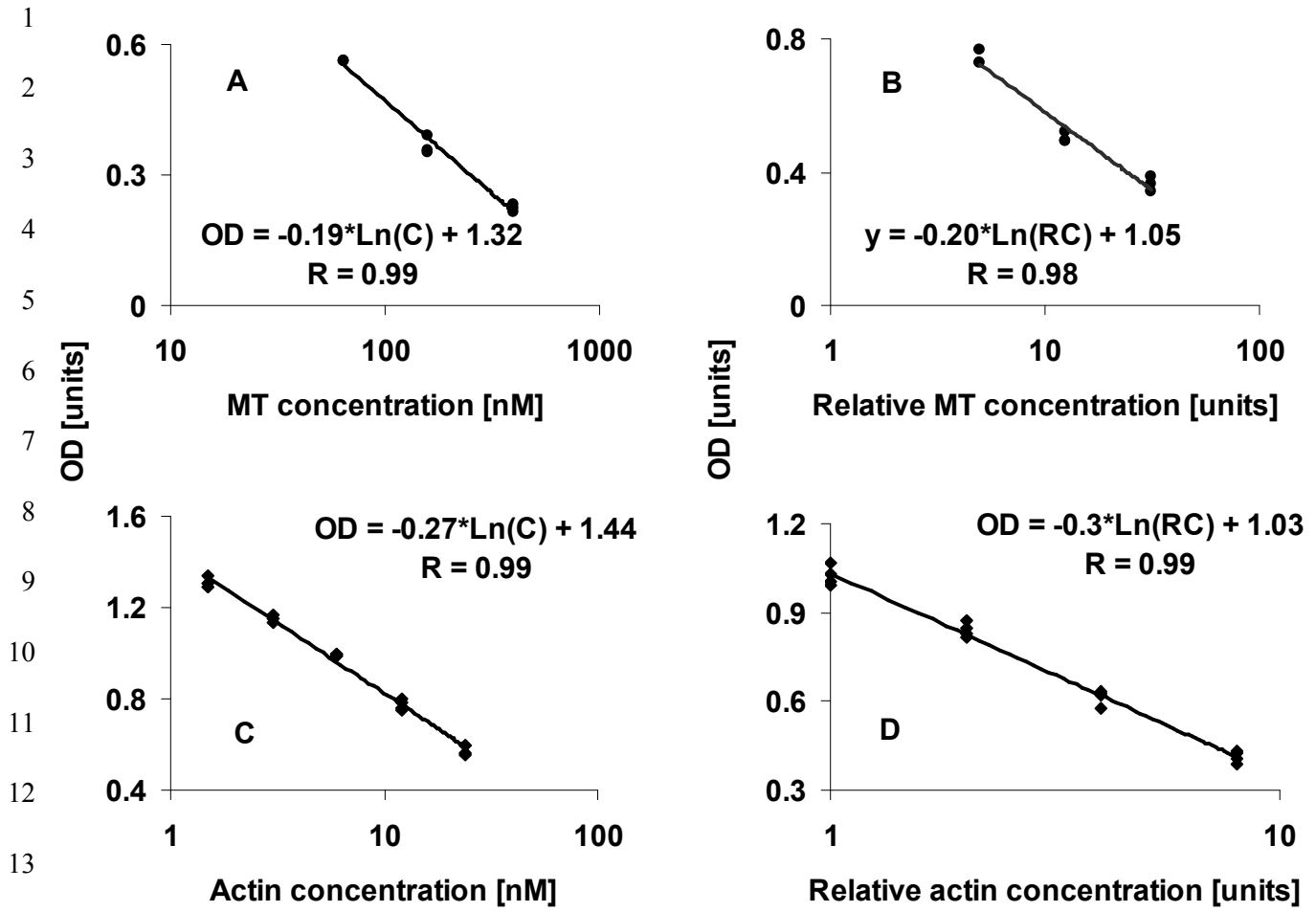


Fig. 3

Fig.4

