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Response of metallothionein gene-1 to laboratory exposure to heavy metals and thermal stress in the freshwater prawn *Macrobrachium rosenbergii*

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ABSTRACT

Metallothioneins, metal-inducible proteins, are being characterized from different organisms and shown as potential biomarkers of exposure to pollution by certain heavy metals. Here we report the identification of a new metallothionein cDNA (433 bp) from the shrimp *Macrobrachium rosenbergii*, putatively encoding a 61 residue polypeptide. Tissue specific analysis indicated that Mar-MT-I (*M. rosenbergii* Metallothionein Gene-1) is expressed with the highest levels in the hepatopancreas and lowest in the thoracic ganglia, and none in the gills or muscles. In addition, our data showed that Mar-MT-I is differentially regulated in the hepatopancreas by certain heavy metals and thermal stress: Cd and Cu produce somewhat similar expression profile patterns, Zn has a reductional effect and thermal stress alone entirely stops its expression. These results show that Mar-MT-I mRNA levels can potentially be used as biomarkers for Cd, Cu or Zn pollution individually. However, in the case of combined metal treatment, different combinations of these metals have quite different effect on Mar-MT-I expression. Therefore, factors of such differential behaviors should be kept as a priority for further biomonitring studies.

1. Introduction

Day by day the environment is being polluted with large number of heavy metals and chemicals, which are dangerous for all living organisms. In these circumstances one key area of focus is the use of biomarkers, as indicators of biochemical change, providing an early warning and assessment of environmental risk. As such bio-chemical markers often parallel changes in the physiochemical characteristics of the environment, the use of biomarkers as detection measures can enable some preventive measures to be adapted in time to avoid certain hazards, particularly applicable to the area of human health. Among the numbers of feasible biomarkers that have been put forward, the Metallothioneins (MTs) and the Hsp70 family have been the ones studied most extensively.

Metallothioneins comprise of a family of ubiquitous, heat stable, low molecular weight (less than 9 kDa), and cysteine-rich (sulfhydryl groups) metal-binding proteins with no aromatic amino acids. Multiple isoforms have been identified and metallothionein polymorphism appears to be a particularly important feature in invertebrates as compared to mammals [1]. Variations in molecular mass have also been observed, suggesting the presence of monomeric and dimeric forms particularly in mollusks [2]. In the past years multiple functions have been attributed to these proteins such as the reception, distribution, storage and release of essential metals (zinc and copper) [3,4] and heavy metal (cadmium and mercury) detoxification [5–7]. MTs have been suggested to be involved in processes such as apoptosis, regulation of neuronal growth, and protection against free radicals and other oxidants [8]. However, in the last decade MTs have been given prime importance in biomarkers studies for the assessment of metal pollution [1,9–11]. Furthermore, MTs have been recognized and examined as a core part of a suite of biomarkers in the framework of biological effect quality assurance in monitoring programmes (BEQUALM) [12].

In polluted environments, when animals are generally exposed to a mixture of different metals and when MT induction is demonstrated, it usually remains impossible to attribute this additional synthesis to any one particular element. In the laboratory, several authors have exposed aquatic organisms to mixtures of metals with the same consequence, remaining unable to give any interpretation regarding the relative roles of each particular element present (rev. from [31]). On the other hand, studies where only a single heavy metal is used to trigger MT response provides little validation of MT as a biomarker and has no ready application to the field as such a situation has little relevance to a real world environmental pollution situation. Studies would be more fruitful and realistic if both parameters are considered simultaneously. Previous literature shows that most of the studies thus far had been conducted on marine animals especially on mussels and crabs. In most of the studies the animals were exposed to high doses of individual heavy metals [9,13–16]

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and less attention was paid to fairly relevant doses of individual metals as well as to their combined effects. At present most of the studies only provide total MT concentrations, despite the fact that different isoforms coexist within organisms [17–20]. Therefore, the consideration of MT mRNA levels as biomarkers of metal pollution could yield more reliable results than simply the isolation of total MT content in exposure to a specific metal because certain studies indicate that less toxic metals are replaced by more toxic one [21,22].

In contrast to marine-life [15,23] there seems a scarcity of information regarding the application of MT mRNA as biomarker for assessment of metal pollution in fresh water animals, especially in shrimps. Therefore, in the present study we isolated and characterized the MT cDNA from the shrimp *M. rosenbergii* and further evaluated its MT mRNA expression levels as a biomarker response for fresh water metal pollution. Because factors including the choice of the best species for monitoring and the choice of the most relevant organ for MT determination remains for further discussion, and the interpretation of any subsequent data may be limited by such factors.

2. Materials and methods

2.1. Exposure to stress and sampling

For the present study both sexes of freshwater prawns, *Macrobrachium rosenbergii*, 15–18 g, were acclimatized for about 1 week in the laboratory before experimentation. They were reared under a 12D–12L photoperiod in a 25 L glass aquarium (16 prawns per aquarium) containing freshwater at 25 °C and fed artificial feedstuff daily. The water was changed after every 2 days. The type of heavy metal, dose concentration, duration and degree of thermal stress is given in Table 1. The gills, hepatopancreas, gut, ovary, thoracic ganglia and muscles were then ablated, snap-frozen in liquid nitrogen and stored at −80 °C for subsequent analysis. Four animals were sacrificed for each sampling.

2.2. Isolation of total RNA and cDNA synthesis

All the samples were homogenized in Trizol Reagent (Invitrogen, USA) and total RNA was isolated according to instructions supplied by manufacturer's kit. The total RNA was quantified on a Genova UV–visible spectrophotometer at 260 nm. Single-stranded cDNA was synthesized from total RNAs using FirstChoice™ RLM-RACE Kit (Ambion, USA), following the instructions supplied by manufacturer's kit.

2.3. Amplification by the polymerase chain reaction (PCR)

A fragment of 182 bp encoding the MT-ORF region was amplified (Fig. 1) from cDNA by using degenerate primers (Table 2). For amplification the PCR conditions were set as: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 30 s and 72 °C for 10 min. Degenerate primers were designed on the basis of highly conserved MT cDNA sequences of *Callinectes sapidus* (AF200418; AF200419; AF200420), *Carcinus maenas* (AF196974; DV467613; DV467269), *Eriocheir sinensis* (AY057396), *Portunus pelagicus* (AY057395), *Scylla serrata* (AY057397), *Pacifastacus leniusculus* (AF199482), *Palaemonetes pugio* (AY935987), *Litopenaeus vannamei* (BF023848) and *Penaeus monodon* (CK991552; AI253912).

Table 1
Dose and duration of exposure to heavy metals and thermal stress.

Metals	Dose (μg/L)	Exposure time (h)
Individual heavy metal treatment		
Cadmium	2.5 and 5	1, 2, 4, 8
Copper	250 and 500	1, 2, 4, 8
Zinc	250 and 500	1, 2, 4, 8
Combined heavy metal and thermal treatment		
Cd + Cu	2.5 + 250	2
Cd + Zn	2.5 + 250	2
Cu + Zn	250 + 250	2
Cd, Cu, Zn	2.5 + 100 + 100	2
Cd, Cu, Zn + 28 °C	2.5 + 100 + 100	2
Heat shock	35 °C	2

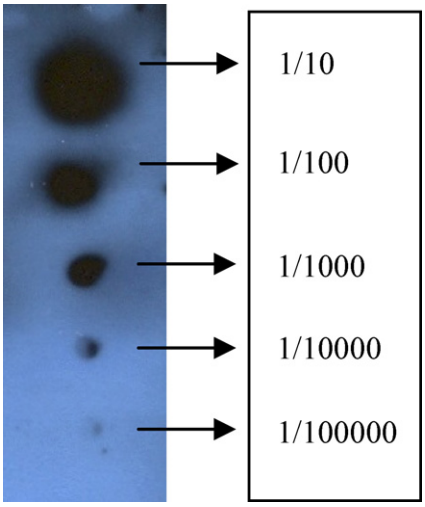

Fig. 1. The hybridization efficiency of Mar-MT probe observed from 1/10 to 1/100,000 dilutions detection of MT cDNA.

2.4. Rapid amplification of cDNA ends (RACE)

The cDNAs for 5′RACE were synthesized from the total RNA using FirstChoice™ RLM-RACE Kit (Ambion, USA), following the instructions supplied by manufacturer's kit. This was later used as a template for 5′RACE PCR. Amplification of 5′RACE was performed in two rounds. The first round was performed by using a gene specific primer (MT-5′RP1) and a Race kit primer (5′ outer P) (Table 2). The PCR conditions were set as: 94 °C for 5 min, 38 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 1.5 min and 72 °C for 10 min. Then in second round of PCR a fragment of about 200 bp (Fig. 1) was amplified by taking template from first round PCR product and using a gene specific primer (MT-5′RP2) and a race kit primer (5′ inner P) (Table 2). The PCR conditions were similar to that of first round except that the primer annealing temperature was set at 54 °C.

The cDNAs for 3′RACE were synthesized from total RNA using FirstChoice™ RLM-RACE Kit (Ambion, USA) following the manufacturers' instructions. All the procedures and components were similar to that of 5′Race with the exception of the use of a 3′ Race adaptor instead of 5′Rcae adaptor. First round of 3′Race PCR was primed by taking the 3′Race product as a template and using a gene specific primer (MT-3′RP1) and a Race kit primer (3′ Outer P) (Table 2). Then in second round of PCR a fragment of about 350 bp (Fig. 1) was amplified by taking template from first round PCR product and using a gene specific primer (MT-3′RP2) and a Race kit primer (3′ Inner P) (Table 2). The PCR conditions for both rounds were same as: 94 °C for 5 min, 38 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 2 min and final extension at 72 °C for 10 min.

2.5. Cloning and sequencing

All products obtained by a TGradient thermocycler (Whatman-Biometra, Goettingen, Germany) were cloned into pUCm-T vectors (Sangon, Shanghai, China). The cDNAs ligated into vector were

Table 2
Primers and adapter used in PCR for the cloning of MT cDNA.

Primer	Direc-tion	Length (bp)	Position	Sequence (5′-3′)
MT-DFP	F	17	96–112	ATGCCWGRYCCMTGCTG
MT-DRP	R	19	266–284	CARCACTTGCAKGSCTTGG
MT-3RP1	F	23	105–128	CCTGCTGTGAAGGAAAGGAAACT
3′ Outer P	R	23		GCGAGCACAGAATTAATACGACT
MT-3RP2	F	20	135–154	TCCAAGGGAGACTGCAAAGG
3′ Inner P	R	32		CGCGGATCCGAATTAATACGACTCACTATAGG
MT-5RP1	R	20	226–245	GAGCACCACGGCAGCAGTTG
5′ Outer P	F	24		GCTGATGGCGATGAATGAACACTG
MT-5RP2	R	20	139–158	GCAGCCTTTGCAGTCTCCCT
5′ Inner P	F	35		CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG
MT-APF	F	18	17–34	CTCAAGTCGCTTCCGTTC
MT-APR	R	22	372–393	TGTCATACAAGATGTTTTCACC
5′-Race adapter				GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA
3′-Race adapter				GCGAGCACAGAATTAATACGACTCACTATAGGT12VN

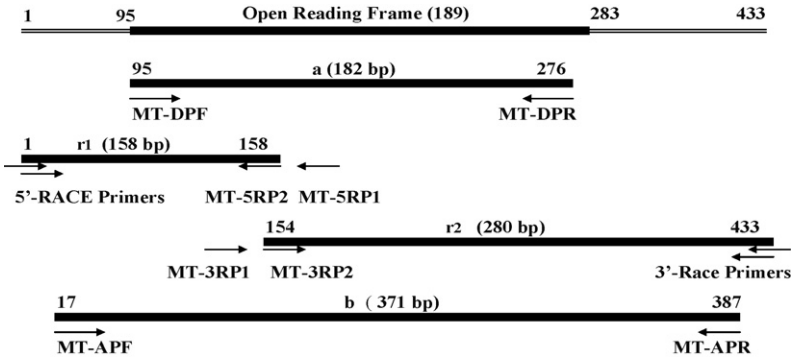

Fig. 2. Schematic diagram of Mar-MT-I cDNA showing locations of primers for polymerase chain reaction (PCR) and cloning strategy of the cDNA. Arrowheads represent primers, and lines above them indicate the cDNA fragments amplified with the primers by PCR (a and b), 5′-RACE (r1), 3′-RACE (r2).

used to transform the competent *E. coli* (DH5-alpha) and were bidirectionally sequenced using the versatile primers M13F/R. All sequencing was performed with ABI PRISM Big Dye terminator chemistry and analyzed on ABI 3730 automated sequencers (Applied Biosystems, Foster City, USA). The nucleotide sequence from another positive clone was determined by independent amplifications (using primers MT-SPF and MT-SPR, Table 2) in order to confirm that the sequence did not harbor any PCR errors (Fig. 1).

2.6. Preparation of probe and Northern analysis

A fragment of 182 bp belonging to ORF of Mar-MT gene was amplified by using primers MT-DPF and MT-DPR (Table 2), and then purified by using a DNA purification kit (TaKaRa Agarose Gel DNA Purification Kit Ver.2.0) following the manufacturer's instructions. The purified PCR product was labeled with digoxigenin-dUTP using the DIG High Prime Labeling Kit (Roche). The probe hybridization efficiency was observed upto 1/100,000 dilution detection of MT cDNA (Fig. 1).

Aliquots containing 10 μg of total RNA corresponding to each sample were mixed with 6 M Glaxol (4 μl), 10X TAE Buffor (2.4 μl) and Deionized DMSO (12 μl). Then denatured at 55 °C and separated through a 1.0% agarose gel, and transferred to a nylon membrane (Millipore immobilon-NYþ), UV cross-linked (CL-508), and then hybridized with the 182 bp MT probe at 42 °C for overnight. The probe hybridization was detected using a DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche). The probe hybridized membrane was exposed to X-ray film for signal detection.

2.7. Statistical analysis

All determinations were performed in triplicate and mean values are presented in the results. Statistical comparisons of the mean

```
AAAACAAGCTCAGCCGCTCAAGTCGCTTCCGTTCGCATCAATTAGTTTGTGCTCGTTCTC    60nt
                                    M  P  D  P  C  C  E  G       8aa
CTGAGTCGTCGAGACCGGTCCCTCTAATCGCCAACATGCCTGATCCATGCTGTGAAGGAA   120nt
K  E  T  D  C  S  K  G  D  C  K  G  C  D  A  G  C  K  G  N      28aa
AGGAAACTGACTGCTCCAAGGGAGACTGCAAAGGCTGCGATGCAGGCTGCAAGGGCAACT   180nt
C  C  R  G  A  P  C  E  K  C  T  P  E  C  A  C  K  S  A  D      48aa
GCTGCCGTGGTGCTCCATGTGAGAAATGCACCCCAGAATGCGCCTGCAAGTCGGCTGATG   240nt
D  C  A  K  N  C  A  K  A  C  K  C  C  P  *                    62aa
ACTGCGCCAAGAACTGCGCTAAAGCCTGCAAGTGCTGCCCATAATGAAATTCAAGTGTTG   300nt
CGAACGTGAATTCTACTAACCTAACTTAACGCGTGTGAGTTTGGATGTCATGAAGTCTTC   360nt
GTTGTGGTGAAAACATCTTGTATGACAGTGAACACTTGAATTCTGAGAGTAAATTATTTC   420nt
TTTAAAAAAAAAA                                                  433nt
```

Fig. 3. The Mar-MT-I cDNA containing 433 bp; with 95 bp in 5′UTR, 189 bp in ORF (putatively encoding 61 residual peptide) and 149 bp in 3′UTR.

values were performed by analysis of variance (ANOVA), followed by Duncan's multiple range test ($p < 0.05$), using SAS 8.3 software (SAS Ins. Inc., Cary, USA).

3. Results

3.1. Cloning and characterization of Mar-MT-I cDNA

The whole cloning strategy is given in Fig. 2, while the nucleotide and amino acid sequence of Mar-MT-I cDNA is given in Fig. 3. The biochemical characteristics showed that the *M. rosenbergii* MT gene is a novel one, with 433 bp cDNA encoding for 62 amino acids residual putative peptide. The 5′UTR, ORF and 3′UTR of MT-I cDNA contains 95, 189 and 149 bp, respectively. The nucleotide sequence was submitted to GenBank/EMBL/DDBJ under the accession number EU871044.

3.2. Mar-MT-I gene expression profile in different tissues

The results showed that Mar-MT-I gene expresses in the hepatopancreas, gut, thorasic ganglia and ovary under normal conditions. Its expression level is higher in the hepatopancreas and less in thorasic ganglia but nil in the gills and muscles (Fig. 4).

3.3. Expression of Mar-MT-I gene under heavy metals and thermal stress

3.3.1. Heavy metals effect on Mar-MT-I gene expression in the hepatopancreas

3.3.1.1. Cadmium. The response of the MT-I isoform to Cd exposure shows that in both of the dose concentrations (2.5 and 5 $\mu g\,L^{-1}$) there is firstly a reduction at 1 h of exposure, then a return to the basal level and then a cycle of increases and subsequent decreases. However such a decrease, increase and again decrease was less in magnitude and also taking more span in cases of low dose exposure (2.5 $\mu g\,L^{-1}$) as compared to high dose (5 $\mu g\,L^{-1}$) exposure. This shows that the MT-I mRNA expression profile pattern is the same under different exposure levels of cadmium. However, variations

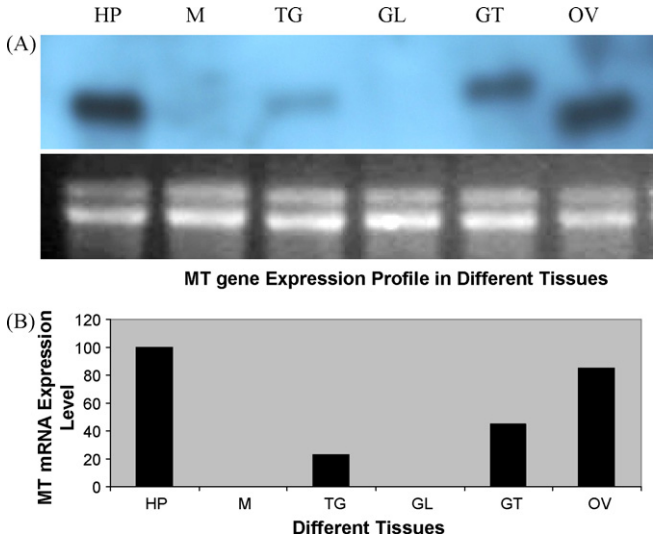

Fig. 4. The analysis of Mar-MT-I gene expression profile in different tissues under normal condition. (A) Northern blot analysis. Total RNAs were analyzed in 1.0% agarose gels, transferred to nylon membranes (Millipore immobilon-NY+) and hybridized to gene specific Mar-MT-I probe. (B) Quantification of the Mar-MT-I expression. Ordinate: the transcripts corrected by the amount of 18 S rRNA and given in arbitrary units. (A and B) Where as HP, M, TG, GL, GT and OV stands for the hepatopancreas, muscles, thorasic ganglia, gills, gut and ovary, respectively. The Mar-MT-I isoform did not show expression in the gills and muscles.

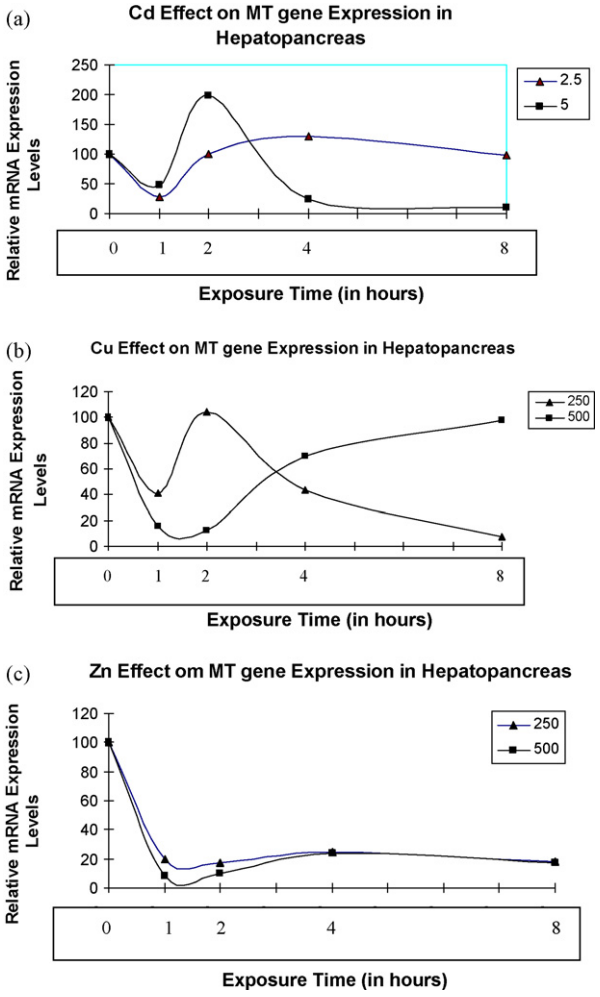

Fig. 5. The analysis of (5a) cadmium, (5b) copper and (5c) zinc effect on Mar-MT-I gene expression in the hepatopancreas. The figure represents the quantification of the Mar-MT-I expression. Ordinate: the transcripts corrected by the amount of 18 S rRNA and given in arbitrary units. The effect of the low dose of Cd, Cu and Zn (2.5, 250 and 250 $\mu g\,L^{-1}$, respectively) is represented by series (–▲–), while that of high dose of Cd, Cu and Zn (5, 500 and 500 $\mu g\,L^{-1}$, respectively) is represented by series (–■–).

exist in mRNA levels with respect to the concentration of dose exposure and the mRNA expression profile pattern which resulted in response to lower dose being achieved in less time in case of higher doses. For example, the maximum expression level was reached after 4 h in exposure to 2.5 $\mu g\,L^{-1}$; whereas the highest level was achieved after 2 h in case of 5 $\mu g\,L^{-1}$ (Fig. 5a).

3.3.1.2. Copper. The effect of copper on MT-I mRNA expression level revealed a significant reduction (about 60%) at the 1st hour and then returned to the basal level reaches (2 h) with the subsequent gradual decrease from 60 to 90% at 4th and 8th hour of exposure to low dose, 250 $\mu g\,L^{-1}$. The response is also somewhat similar in case of high dose exposure, 500 $\mu g\,L^{-1}$; where it caused a significant reduction (85 to 90%) in the expression levels at 1st and 2nd hours of exposure and then gradually increased to 70% to almost basal level at 4th and 8th hour of exposure, respectively (Fig. 5b).

The expression of Mar-MT-I isoform in exposure to Cu also adopts a somewhat similar pattern (Fig. 5b) when compared to that of Cd (Fig. 5a) where in both doses of Cd and Cu, for example, there is considerable reduction in the 1st hour of exposure. However, the time span for returning to the basal level differs between different doses of both metals. For example, the basal level was achieved for

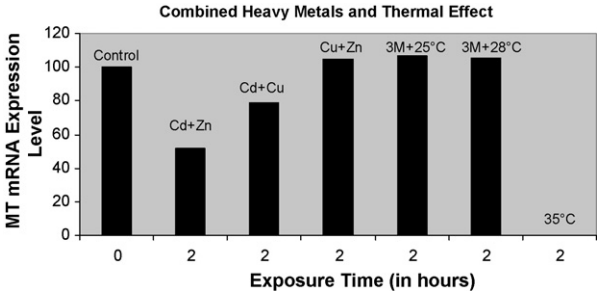

Fig. 6. The analysis of Mar-MT-I gene expression in exposure to different combinations of heavy metals. The figure represents the quantification of the Mar-MT-I expression. Ordinate: the transcripts corrected by the amount of 18 S rRNA and given in arbitrary units. From left to right: Control (no treatment), Cd + Zn ($2.5 + 250\ \mu g\ L^{-1}$), Cd + Cu ($2.5 + 250\ \mu g\ L^{-1}$), Cu + Zn ($250 + 250\ \mu g\ L^{-1}$), Cd + Cu + Zn ($2.5 + 100 + 100\ \mu g\ L^{-1} + 25\ ^{\circ}C$), Cd + Cu + Zn + heat shock ($2.5 + 100 + 100\ \mu g\ L^{-1} + 28\ ^{\circ}C$) and thermal stress (35 °C) alone for 2 h exposure.

longer time in case of $2.5\ \mu g\ L^{-1}$ of Cd as compared to $250\ \mu g\ L^{-1}$ of Cu during 8 h of exposure.

3.3.1.3. Zinc. The effect of zinc on MT-I mRNA expression level showed that different concentrations ($250\ \mu g\ L^{-1}$ and $500\ \mu g\ L^{-1}$) of zinc are able to cause a considerable reduction (about 80 to 90%) in basal levels. However, such a reduction was comparatively a little bit more in the former stages (1st and 2nd hour) in the case of a high dose exposure and beyond that (4th and 8th hour) both doses have almost the same reductional effect (Fig. 5c). These results show that Zn suppresses the normally expressing MT-I isoform and simultaneously most probably also causes the induction of another MT isoform (which may be called Zn induced MT isoform). As zinc is also an essential nutrient so there must be a storage/detoxification mechanism for its excess. Though the confirmation of this thing needs further research work, but our assumption is also strengthened by a couple of earlier studies that two to three MT isoforms are present in invertebrates including crustaceans [18,20,23,27]. Therefore, the possibility of another MT isoforms responding to zinc is not uncertain.

3.3.1.4. Combined heavy metal and thermal stress. In case of combined metal treatment both the Cd + Zn ($2.5 + 250\ \mu g\ L^{-1}$) and Cd + Cu ($2.5 + 250\ \mu g\ L^{-1}$) showed a reductional effect on MT mRNA levels. However, such reduction was observed at a higher level (about 50%) in response to Cd + Zn combination as compared to Cd + Cu (about 20%). The combination of Cu + Zn ($250 + 250\ \mu g\ L^{-1}$) and Cd + Cu + Zn ($2.5 + 100 + 100\ \mu g\ L^{-1}$) at 25 °C, and Cd + Cu + Zn ($2.5 + 100 + 100\ \mu g\ L^{-1}$) at 28 °C showed an insignificant effect when compared to basal mRNA levels. However, heat shock of 35 °C alone suppressed the induction of MT mRNA levels to a non-detectable level (Fig. 6). These induction responses were noted for 2 h of exposure.

3.3.2. Heavy metals effect on MT-I gene expression in the gills

We failed to observe MT induction of present isoform in the gills in response to heat shock or to different heavy metal (cadmium, copper and zinc) concentrations tested singly as well as in combination for 0–8 h exposure.

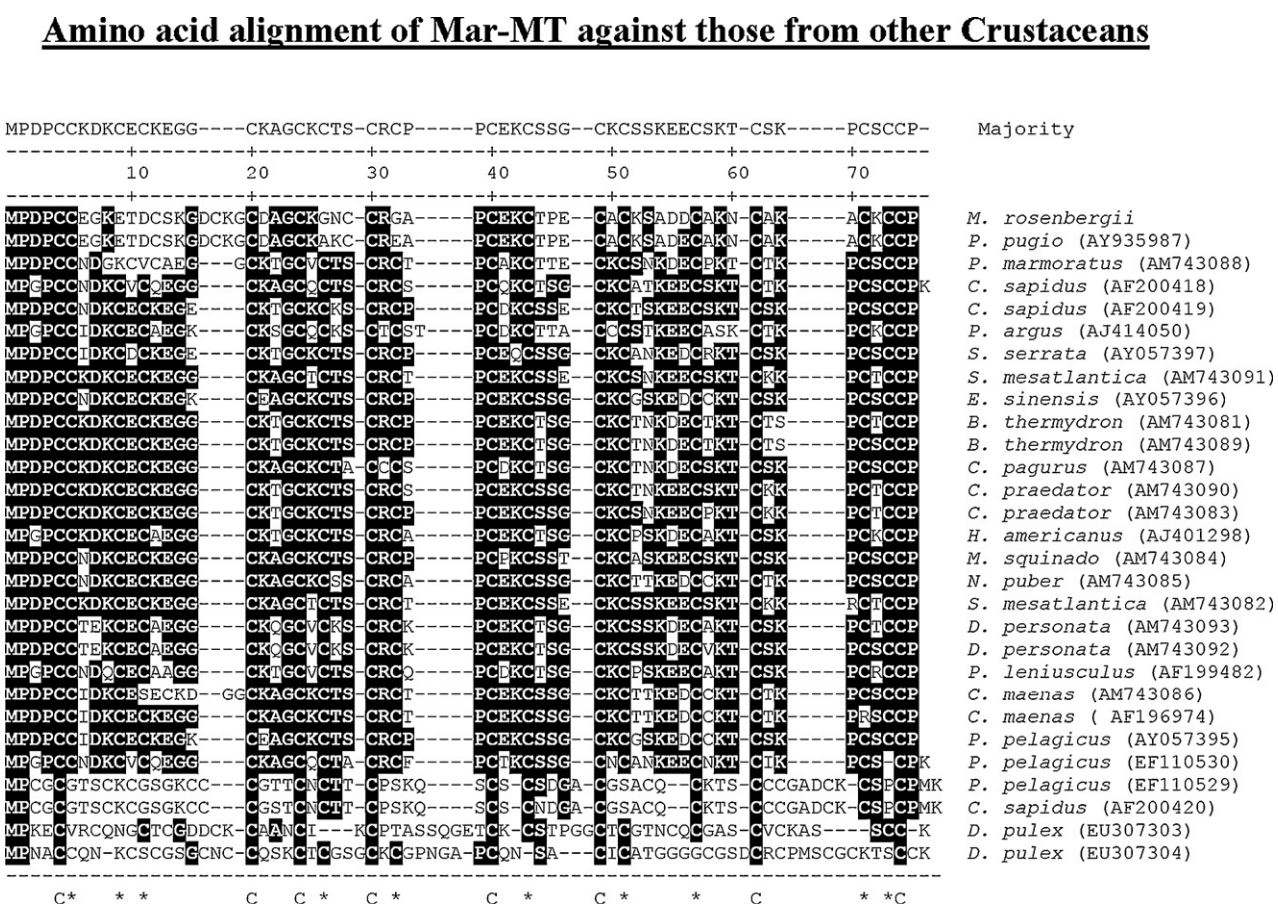

Fig. 7. A comparison of amino acid alignment of *Macrobrachium rosenbergii* metallothioneins protein plus 29 sequences from 19 selected species. The NCBI accession numbers are listed within brackets with each taxonomic gene designation.

4. Discussion

4.1. Cloning and Characterization of MT cDNA

MTs are involved in heavy metal detoxification [6,7,24] in addition to storage of essential elements that are necessary for metalloenzymes [17,25]. Such a functional homology of MTs in the diversity of organisms is based on the specificity of their content as well as on their structural arrangement. The most important characteristic of MTs is not only the presence of high cysteine content but also the conservation of its position in the architecture of the MT molecule. The additional incorporation or the replacement of certain amino acids may cause variability in metal-binding ability and thus affecting the mechanism and role of MT in heavy metal detoxification. The comparison of different Crustaceans MTs (including 29 sequences belonging to 19 species) with that of the Mar-MT-I (*M. rosenbergii* MT) peptide sequence has shown that where most of crustacean MTs contain 58 amino acid residues and a few, including Mar-MT-I, have some additional amino acid residues that had been incorporated during the course of evolution. Such insertions are given in detail in Fig. 7. The amino acid alignment also showed that five proline residues and one argenine are highly conserved in addition to cysteine residues. Among the cysteine content, 8 represented by "C" are conserved in these species, while other 10 cysteines represented by "*" show conservation degeneracy. Furthermore, cysteines are also regarded as domin partitions and metal-binding residues [26,27]. Therefore, we have aligned the MT peptides of different crustaceans for comparison on the basis of cysteine residues.

In the case of *M. rosenbergii* 17 cysteines are present instead of 18, which exist in the majority of species. Another novelty in Mar-MT-I is presence of 4 additional amino acids DCKG as is the situation for *P. pugio*. Furthermore, 16 amino acid residues at different positions in the Mar-MT-I peptide are not found in any other species at the same position, with the exception of *P. pugio*. However, Mar-MT peptide varies from that of *P. pugio* at position 26, 27, 32, and 56 in having GNGD amino acid residues instead of AKEE, respectively. The BLAST search showed that sequence identity of Mar-MT-I with other crustaceans ranges from 24% (with *Daphnia pulex*, EU307303) to 93% (with *P. pugio*, AY935987) with an average 66%. Thus Mar-MT-I is a novel peptide among crustacean MTs.

4.2. Mar-MT-I gene expression profile in different tissues

Invertebrate metallothioneins have been mainly reported in digestive tissues [4,5,11] gut, nervous tissues, muscles [5], kidney, heart and gills [28], but data about the existence of a MT isoform expressed also in the ovary, is not available. Here we report the identification of a new metallothionein gene (Mar-MT-I) from the *M. rosenbergii* expressed in the hepatopancreas, ovary, intestine and nervous tissue, with the highest levels in the hepatopancreas and the lowest in the nervous tissue (thorasic ganglia) but no detectable expression in the gills and muscles (Fig. 4). Its expression in the ovaries reveals the importance of MT in handling the essential metabolic elements during the developmental process. The size of MT molecule expressing in the gut seems to be a little bit larger than that of other tissues. Such size variations have also been seen in *Daphnia pulex* [19].

4.3. Expression of Mar-MT-I gene under heavy metals and thermal stress

4.3.1. Hepatopancreas

In various studies the MT content was isolated and measured in response to heavy metal exposure [11,20,29]. Therefore, metallothionein had been extensively studied as a biomarker of metal pollution [12,30]. It is also well known that different isoforms of MTs exist in the living beings [17,18,19,20]. The problem associated with isolation of total MT content lies in the difficulty of differentiation between old and newly synthesized specific MT isoform. As a couple of studies have shown the tendency for less toxic metals e.g. zinc to be replaced by more toxic ones [21], and this factor would have a strong confounding effect on the interpretation of results. Similarly, Ni ions have a very high affinity for cysteine [22]. Furthermore, the concentrations of compounds which share many characteristics of MT (cytosolic, heatstable, thiol-rich compounds–CHSTC) effect the results by an overvaluation of the true MT concentration [6]. Therefore, keeping such problems in mind we studied the induction of the MT gene in response to heavy metal exposure in *M. rosenbergii* through northern analysis, because the northern analysis in such circumstances yields better information about the induction of a specific isoform of MT.

The comparison of results with the species from different habitats for biomarkers studies could be suggested to be logically flawed and data can be compared strictly only when they have been obtained with the same analytical technique. Studies on MT gene induction are very few; while in most of the cases the stress was emphasized on total MT isolation for the assessment of metal pollution [31]. In a number of studies the animals were exposed to the high dose concentrations of Cd and in some cases to Zn also (see introduction). In our study we have used the dose concentrations within limits of EPA standards.

However, the sensitivity, quickness and metal specificity of gene response can be compared to elucidate the usefulness of a species for biomarker studies. In the literature we found one study in crustacean on MT gene expression in *Panulirus argus* [5]. In the case of Zn our results are in agreement with this study that the doubling of a Zn dose has insignificant effect ($p \geq 0.05$) on MT mRNA levels in crustaceans. However, with respect to sensitivity of response the Mar-MT-I gene is much more sensitive than MTPA (Metallothioneins *P. argus*) because 25 and 250 μM Cu and 250 μM Cu and 250 μM Zn had shown insignificant effect ($p \geq 0.05$) on MTPA mRNA levels. Furthermore, 25 μM Cd is much toxic than 250 μM Zn but both showed an insignificant effect ($p \geq 0.05$) on MTPA mRNA levels [5]. While in our case there is a significant effect ($p < 0.05$) on mRNA levels between Cu and Cd as well as between their different doses, and the effect of Zn can also be measured in a severe reduction in the mRNA levels. Apart from sensitivity, the response of Mar-MT-I is also very quick and thus results can be achieved within hours instead of several days as compared to other species [31]. Our results show some contradiction with the study conducted on the amphipod *Echinogammarus echinosetosus*. This showed significant ($p < 0.05$) MT induction to a range of Cd concentrations from 100 to 1000 μg/l for a period of 24 h exposure [32], where as the increasing or decreasing levels of Mar-MT-I were not dependent on time and dose concentration in both cases of heavy metals (Cd and Cu).

Our study also shows that MT response in exposure to individual heavy metals is quite different from that of combined heavy metals. For example, separately exposure to 2.5 $\mu g\,L^{-1}$ Cd (Fig. 5a) and 250 $\mu g\,L^{-1}$ Cu (Fig. 5b) has shown an insignificant effect ($p \geq 0.05$) on the basal levels for a 2 h exposure; while the same concentrations of these two metals in combination has shown reduction, though insignificant (about 20%), in basal levels. In such case the Cd-induced isoform has possibly also expressed. On the other hand, 250 $\mu g\,L^{-1}$ Zn has shown significant ($p < 0.05$) reduction (about 80%) in the basal levels, while in combination with 2.5 $\mu g\,L^{-1}$ Cd it has shown only about 50% reduction in the basal levels (Fig. 6). These results show that Zn alone probably causes the induction of another specific isoform and in combination with Cd the both Zn-and Cu-induced isoforms are expressed equally. This assumption is strengthened by insignificant effect ($p \geq 0.05$) on the basal levels by Cu + Zn (250 + 250 $\mu g\,L^{-1}$) combination, showing that Cu-

induced isoform is already expressing and Zn has induced another isoform. Furthermore, it also seems that the nucleotide sequence of these (Zn- and Cu-induced) isoforms is considerably different from each other. As zinc is also an essential nutrient so there must be a storage/detoxification mechanism for its excess. Though confirmation of this assumption needs further investigation, but our assumption is also supported by a couple of earlier studies that two to three MT isoforms are present in invertebrates including crustaceans [18,20,23,27]. Therefore, the possibility of another MT isoforms responding to zinc is not uncertain.

Our results have also shown that thermal stress has no effect on Mar-MT-I induction when there is also metal stress [Fig. 6, lane 5 (three metals + 25 °C and lane 6 (three metals + 28 °C). However, thermal stress (35 °C) alone stops expression completely (Fig. 6, lane 7). This finding is in agreement with earlier studies that the genes expressing under normal conditions are switched off during heat shock response [33]. Thus Mar-MT-I response in exposure to individual heavy metals is quite different from that of combined heavy metals and for the application of MT mRNA as biomarkers for assessment of metal pollution the behavior of all the MT isoforms of an organism should already be known. For example, in the *Callinectes sapidus* MT-I is inducible by Cd, Zn, and Cu; MT-II, inducible by Cd and Zn; and MT-III, inducible by Cu only [18]. With these *Callinectes sapidus* MT isoforms, the Mar-MT-I having the highest sequence similarity with MT-I showed the induction by Cd and Cu but not by Zn. This shows that response of specific MT isoform varies from organism to organism. Therefore, strictly single metal specific MT isoforms, such as MT-III in *Callinectes sapidus* [18], should be explored and then an application of their mRNA levels is much valuable for such purpose.

4.3.2. Gills

We remained unable to locate any reference to studies with the lack of MT gene expression in the gills of crustaceans, while a number of studies show the presence of MT peptides in the gills [11,16,29,34]. As the gills are the utmost in facing the heavy metal stress, so question arises about their antistress mechanism against metal toxicity other than Cu. However, the survival of prawns to MCLs (maximum contamination limits) (set by EPA- Environmental Protection Agency) of Cd and Zn, but not to that of Cu, shows that either there is expression of a Cd/Zn specific MT isoform or the MCLs of these two metals are optimum. However, to answer such questions the gills of *M. rosenbergii* should be studied further for the existence of other MT isoform.

According to the best of our knowledge, it is first report about the lack of a MT isoform expression in the gills of a crustacean, *M. rosenbergii*. The significance of such a finding lays in the study of Hsp70 family as biomarkers of toxicity and effect because the physiological mechanisms dealing with the detoxification of heavy metals may act as confounding factors for the assessment of toxicity and its effect through the Hsp70 family. Therefore, tissue such as gill tissue, lacking MT gene expression and equipped with Hsp70 gene expression (Liu et al. [35] is very useful for such application. Furthermore, such information is important for aquaculture farmers with regards to use of copper for controlling the water blooming.

5. Conclusion

The application of MT mRNA levels yields better information regarding metal pollution than isolation of MT content and the factors such as differential behaviors of MTs with regards to individual and combined heavy metals should be kept as a priority for further biomonitring studies. Furthermore, the gills of *M. rosenbergii* may be suitable for the study of Hsp70 family as biomarkers of toxicity, especially in case of heavy metals.

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