Helgol Mar Res (2002) 56:95–101
DOI 10.1007/s10152-002-0107-1

ORIGINAL ARTICLE

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Multiple forms of metallothionein from the digestive gland of naturally occurring and cadmium-exposed mussels, *Mytilus galloprovincialis*

Received: 15 August 2001 / Revised: 4 April 2002 / Accepted: 4 April 2002 / Published online: 22 May 2002

Abstract Polymorphism of metallothioneins in the digestive gland of naturally occurring (control) and experimentally Cd-exposed mussels *Mytilus galloprovincialis* (200 µg Cd l^{-1}; 14 days) was studied by applying the conventional methods of Sephadex column liquid chromatography (G-75 and DEAE A-25), and by an electrochemical method (DPASV) for determination of Cd, Zn and Cu concentrations in chromatographic fractions. In both control and Cd-exposed mussels, two distinct molecular mass components of the metallothioneins, monomeric (MT-10) and dimeric (MT-20), were resolved by Sephadex G-75 gel filtration chromatography. In control mussels, the MT-10 component was predominantly expressed as containing markedly higher constitutive levels of Zn (100×) and Cu (10×) than of Cd. Each of these two molecular mass components was further resolved into seven metal-rich peaks by anion-exchange chromatography. In Cd-exposed mussels the larger proportion of Cd was bound to the MT-20 than to the MT-10 component, suggesting that the dimeric component may be considered as a primarily inducible metallothionein. The elution positions of metal-binding maxima of Cd-exposed and control mussels on the respective DEAE chromatographic profiles were comparable. A great similarity in elution positions of Cd maxima between the composite and single-specimen samples was also observed. Our study confirms a high multiplicity of MT forms in mussels from the *Mytilus* genus not only under the laboratory high-level metal exposure conditions, but also at a natural seawater metal exposure level. The ecotoxicological significance of dimeric and monomeric MT forms, as well as their possible application in the biomonitoring of seawater for trace metals, has been considered.

Communicated by H.-D. Franke

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Keywords Metallothionein · Isoforms · *Mytilus galloprovincialis* · Digestive gland

Introduction

Polymorphism of the metallothionein (MT) family is a well-known phenomenon frequently observed in marine invertebrate species (Dallinger et al. 1989; Brouwer et al. 1995). Although the biological role of such inducible, low molecular mass, cysteine-rich, metal-binding proteins has not yet been entirely defined, the multiplicity of MT isoforms may be attributed to several proposed physiological and protective functions particularly dealing with trace-metal metabolism, presumably related to their distinct thermodynamic and kinetic properties (Otvos et al. 1993). So far, among all invertebrate species investigated, the blue mussel, *Mytilus edulis*, possesses the largest number of MT isoforms (Mackay et al. 1993). By comparison, in the Mediterranean species of the same genus, *Mytilus galloprovincialis*, a considerably smaller number of MT charge-distinct forms has been identified in selected organs and tissues (Carpenè et al. 1983; Viarengo et al. 1984; Pavičić et al. 1991). In general, two molecular mass classes of MT, monomeric (MT-10) and dimeric (MT-20), were determined in soft tissues of both species experimentally exposed to elevated levels of cadmium or mercury (George et al. 1979; Frazier et al. 1985; Roesijadi 1986). Further fractionation by conventional ion-exchange (DEAE) chromatography, or by reverse phase HPLC, reveals the high complexity of each mass class, particularly in *M. edulis*, being resolved in several charge-distinct components, putative isoforms.

Most studies of MT isoforms in mussels involved their characterization during metal exposure experiments, but there is very little data available concerning MT expression in wild mussels collected from the environment. Based on the fact that MTs are used as biomarkers of heavy-metal pollution, some knowledge of the MT expression in native populations of mussels may be very

important to any practical application of this biochemical indicator.

The purpose of this study was to confirm the existence of multiple MT forms in the digestive gland of naturally occurring and Cd-exposed mussels, *M. galloprovincialis*. Furthermore, our intention was to find out whether a similar pattern of MT multiplicity may exist in the digestive gland of a single mussel when compared with the sample composed of a larger number of mussels, having in mind a possible contribution of allelic polymorphism within mussel populations.

Methods

Exposure conditions

Adult organisms of *M. galloprovincialis*, 5–7 cm shell length, were collected at the rearing station in the Lim Channel (western Istrian coast, north Adriatic), which is considered to be a metal-unpolluted area. After several days of acclimatization to laboratory conditions, the organisms were exposed for 14 days to an elevated level of cadmium (200 µg l^{-1}) in an open continuous flow-through seawater system. Following the exposure period, the digestive glands from 15–20 mussels were dissected on an ice bath in order to prepare the composite samples in addition to several individual samples containing the digestive gland of a single specimen. The samples were kept at –80°C until further treatment. Control, unexposed mussels were collected at the same location in the Lim Channel.

Sample preparation

The tissue was homogenized in a 3:1 volume ratio of buffer (0.02 M Tris-HCl, pH 8.6) containing 5 mM dithiotreitol (DTT) and the protease inhibitors (0.5 mM phenyl-methyl-sulphonilfluoride, PMSF and 0.006 mM leupeptin). The homogenate was centrifuged at 30,000 *g* and 4°C for 45 min.; the soluble phase was used for the subsequent two MT-separation steps by conventional column liquid chromatography.

Isolation and partial characterization of MT

All chromatographic procedures were carried out at 4°C, using the buffer de-aerated with nitrogen. The initial separation step was performed on a Sephadex G-75 gel-filtration column previously equilibrated with a 0.02 M Tris-HCl buffer, pH 8.6 containing 1 mM DTT, as previously reported by Pavičić et al. (1987, 1989).

Chromatographic fractions were collected, and the absorbances at 254 and 280 nm recorded. The concentrations of cadmium, copper and zinc were measured in the chromatographic fractions isolated from the wild mussels, while only Cd concentration was determined in the chromatographic fractions from the Cd-exposed mussels. Those fractions eluting in the MT region (the middle part of the Sephadex G-75 elution profile corresponding approximately to the range of the apparent molecular masses from 7,000 to 25,000) characterized by high absorbance at 254 nm and relatively high metal content, were pooled as MT monomer and dimer. Thus, from each gel-filtration chromatographic run, two samples for further MT fractionation by conventional ion-exchange chromatography were prepared and designated as MT-20 and MT-10 (putative MT dimers with molecular mass of 20,000, and MT monomers of 10,000, respectively; see Fig. 1).

Pooled fractions MT-20 and MT-10 were applied separately to a DEAE-Sephadex A-25 column (1.5×6 cm), which was previously equilibrated with the 0.02 M Tris-HCl buffer, pH 7.4, containing 1 mM DTT, and eluted by an increasing concentration gradient of NaCl (0–0.6 M) in the same buffer.

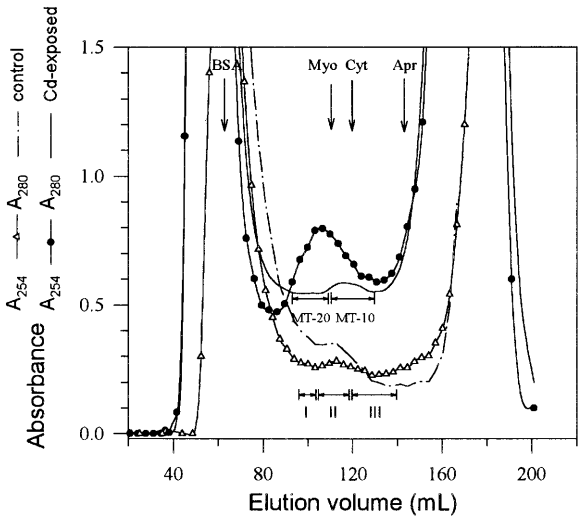

Fig. 1 Comparative presentation of Sephadex G-75 gel-filtration profiles of digestive-gland 30,000 *g* supernatant from control and Cd-exposed mussels. *MT-20* and *MT-10* horizontal bars show the fractions pooled for further ionic-exchange chromatography. *I, II* and *III* indicate the fractions pooled for metal determination. Elution positions of standard molecular mass markers are also given: *BSA* (bovine serum albumin; 66,000), *Myo* (myoglobin; 17,800), *Cyt* (cytochrome C; 12,300), *Apr* (aprotinin; 6,500)

Metal determination

Metal concentrations (Cd, Zn and Cu) in chromatographic fractions were determined electrochemically using a differential pulse anodic stripping voltammetry (DPASV) on the hanging mercury drop electrode by applying the standard addition method. The procedure of metal determination has been described in detail by Raspor et al. (1989, 1998).

Results

The chromatographic profile of the preliminary purification step of MT by gel-filtration column (Sephadex G-75) is presented in Fig. 1. The UV-absorbances at 254 and 280 nm recorded in the samples from the digestive gland of Cd-exposed and control mussels are shown. Obviously, a higher level of MT was isolated from Cd-exposed mussels than from the control specimens of the same population. The ratio of A_{254}/A_{280} within the MT-fractionating region is significantly higher in Cd-exposed than in control mussels. Furthermore, in Cd-exposed mussels, a considerably higher proportion of the dimeric component was observed compared with the control mussels, which contained a larger proportion of the monomeric component.

Metallothioneins in naturally occurring mussels

The results of metal distribution in the pooled chromatographic fractions, designated as regions I, II and III on the Sephadex G-75 gel-filtration profile in Fig. 1, are presented in Table 1. In the soluble phase of the digestive

Table 1 *Mytilus galloprovincialis*, digestive gland 30,000 *g* supernatant of control mussels. Concentrations of Cd, Cu and Zn determined in aliquots of the pooled chromatographic fractions corresponding to regions I, II and III designated on Sephadex G-75 gel-filtration profile in Fig. 1

Pooled fractions	Ve (ml)	Molecular mass (kDa)	Cd (10^{-8} mol/l)	Cu (10^{-7} mol/l)	Zn (10^{-6} mol/l)
I (MT-20)	100	23	1.9	3.81	2.14
II(MT-20 and MT-10)	110	17	6.25	7.49	2.22
III (MT-10)	130	10.4	2.37	4.83	1.68

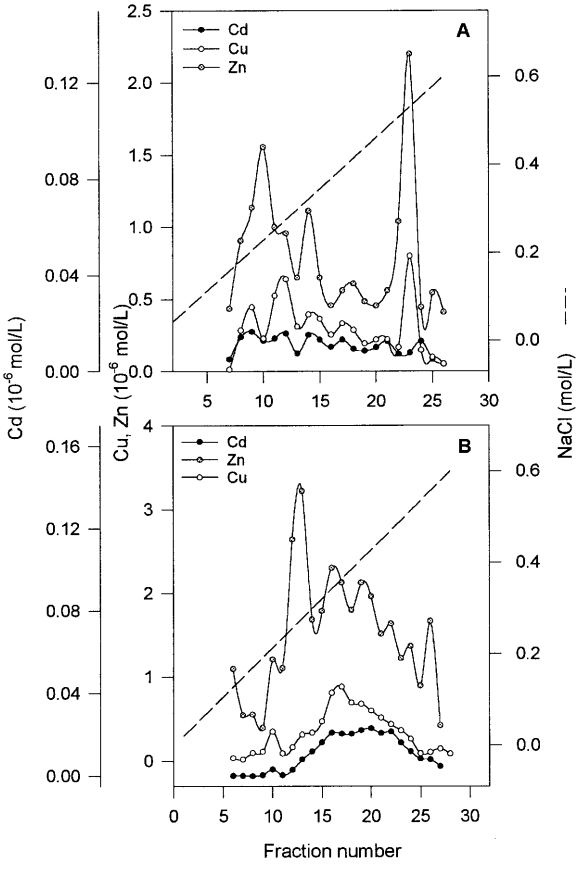

Fig. 2 Metal distribution in DEAE-Sephadex A-25 profiles of MT-20 (**A**) and MT-10 (**B**) components obtained after Sephadex G-75 chromatography of digestive-gland supernatant from control mussels

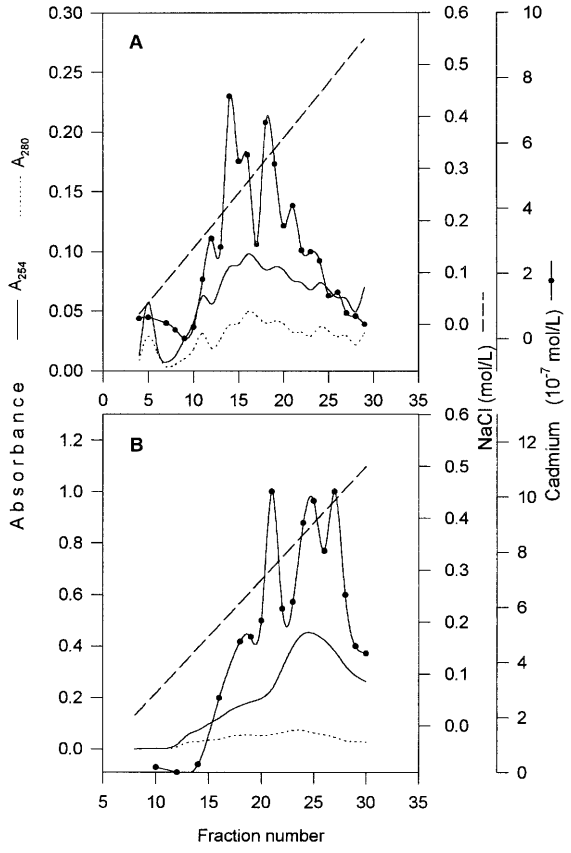

Fig. 3 DEAE-Sephadex A-25 profiles of MT-20 (**A**) and MT-10 (**B**) components obtained after Sephadex G-75 chromatography of digestive-gland supernatant from Cd-exposed mussels (composite sample)

gland, Zn and Cu occurred at levels of about 100× and 10×, respectively, higher than Cd. Within region I, dimeric MT-20 was the main component; followed by a region II, containing mixed MT-20 and MT-10 components; and region III, containing predominantly the monomeric MT-10 component.

Further separation of the MT-20 component by anion-exchange chromatography revealed at least six metal-rich peaks (Fig. 2A), five of them containing predominantly Zn and Cu and with a low level of Cd. It can be noticed that among three prominent Zn-binding peaks, the one eluting at 0.23 M NaCl contains exclusively Zn. The two other peaks beside Zn also contain Cu and Cd. We assume that all these charged forms may not be attributed exclusively to the dimeric MTs, but instead to some mixture of dimeric and monomeric components. On DEAE-elution profile (Fig. 2B) of the MT-10 component, seven Zn-containing peaks were observed, having a variable amount of Cu and Cd. The most prominent Zn peak, which elutes at 0.28 M NaCl, contained low Cu and Cd levels.

Metallothioneins in Cd-exposed mussels

On the Sephadex G-75 elution profile (Fig. 1), Cd-binding proteins isolated from the digestive gland of Cd-exposed mussels occur as two molecular mass classes of about 20,000–25,000 (MT dimer) and 10,000–14,000 (MT monomer), respectively. However, it is important to point out that a higher level of Cd is associated with MT dimer in comparison with MT monomer.

From the Sephadex-purified MT-20 fraction, three major and four minor Cd-containing peaks were resolved by DEAE anion-exchange chromatography (Fig. 3A). Each cadmium peak coincided with an UV-absorbance

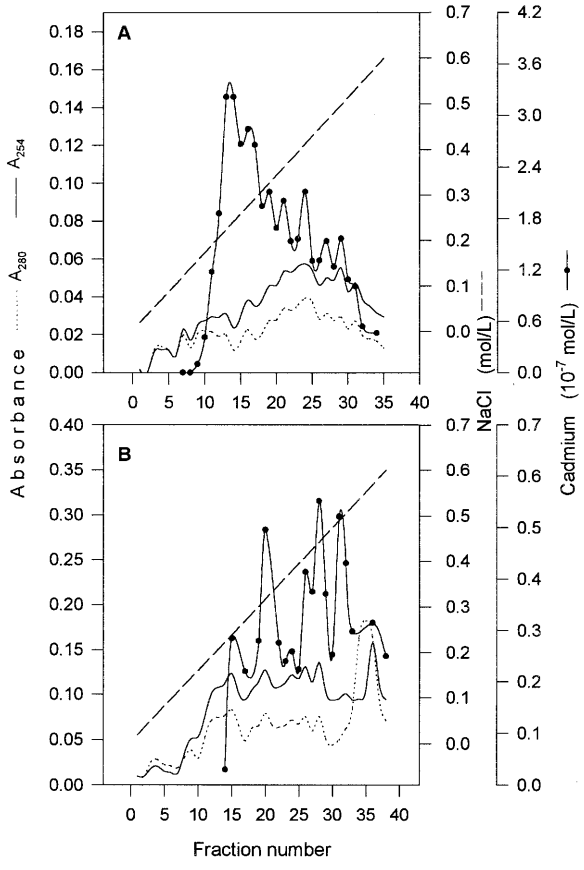

Fig. 4 DEAE-Sephadex A-25 elution profiles of MT-20 (**A**) and MT-10 (**B**) components obtained after Sephadex G-75 chromatography of digestive-gland supernatant from a single Cd-exposed mussel

peak, showing the specific spectroscopic properties when Cd-MT is present ($A_{250}/A_{280}>1$). On the DEAE elution profile of the MT-10 fraction, four Cd-containing peaks were observed (Fig. 3B).

Metallothioneins in a single Cd-exposed mussel

Further separation of the Sephadex G-75-purified MT-20 and MT-10 components originating from a single Cd-exposed mussel by DEAE anion-exchange chromatography resulted in the resolution of six and seven cadmium peaks, respectively (Fig. 4A, B). The elution positions of these Cd-binding components were basically similar to those derived from the composite mussel sample (i.e. a sample composed of 20 mussels), although a greater number of Cd peaks was observed on the MT-10 DEAE elution profile of the single Cd-exposed mussel.

Discussion

Our results on MT components isolated by the conventional liquid chromatography techniques from the digestive gland of Cd-exposed mussels (*M. galloprovincialis*) are generally in agreement with the literature data previously reported for *M. edulis* (George et al. 1979; Carpenè et al. 1983; Frazier et al. 1985; Mackay et al. 1993). The authors report the existence of two molecular-mass classes which are separable by the conventional gel-filtration (Sephadex G-75) chromatography. They were designated as MT-10 (monomer) and MT-20 (dimer), following the terminology proposed by Mackay et al. (1993). Further purification on a DEAE-Sephadex A-25 column indicates a great complexity of each molecular mass class which were resolved into several distinctively charged MT forms, similarly as previously demonstrated in *M. edulis* species (George et al. 1979; Frazier et al. 1985). The functional significance of that large number of multiple forms has still not been fully explained. Bearing in mind only partial characterization of isolated metal-binding components and limited gene analysis data in marine invertebrates, it is preferable to use the term multiple MT forms instead of isoforms, which is widespread in the previously published reports. According to the adopted biochemical nomenclature (IUPAC-IUB CBN 1976) and in agreement with the recently proposed MT classification (Kojima et al. 1999), the term isometallothionein should be attributed to MT forms arising from the genetically determined differences in their primary structure. Other MT variants possibly modified on post-translation level by N-terminal acetylation (Roesijadi 1994) should not be called isoforms (Berger et al. 1995).

Summarizing the earlier literature available on MT multiple forms in *Mytilus* spp. and other bivalve species, with a particular emphasis on the molecular mass determination, we may notice that the majority of data are in accordance with the statement that dimeric forms of MTs, both in mammals and in invertebrates, were mostly produced in organisms either by oxidation following exposure to excess metal ions (Suzuki et al. 1983), or by S–S bridge formation during the procedure of sample preparation (Minkel et al. 1980). Later on, it has also been suggested that these oxidative mammalian MT products were not the same as the native dimeric species proposed to be in continuous reversible equilibrium with MT monomers (Otvos et al. 1993).

The existence of a possibly different type of MT dimer in *M. edulis*, presumably independent of S–S bridging, was first suggested by Frazier et al. (1985), and was later confirmed by amino acid sequential analysis (Mackay et al. 1993) on the basis of which the unique structure was proposed by forming dimers through intermolecular metal-bridging between two sulphur atoms (designed as S–M–S) with an additional Cd ion inserted. Consequently, the authors proposed an increasing metal complexing capacity and a possible distinction in mobility between such bridging Cd and of the Cd ions incorporated into cluster structure. The results presented here suggest the existence of both oxidative S–S and Cd-bridging S–M–S dimers, the latter being irreversible in the presence of reductive agents (dithiotreitol, 2-mercaptoethanol). So far, the physiological or protective significance of those

Table 2 A survey of investigations on MT isoforms in several bivalve species

Organism	Exposure conditions	Organs/tissue	Isoforms	Methods	Reference
Mytilus edulis	Cd 0.1 mg/l 3 months	Digestive gland	3 (MT-20)	DEAE–cellulose	George et al. (1979)
Mytilus edulis	Cd 0.1 mg/l 3 months	Mantle	3 (MT-20)	DEAE–cellulose	Carpenè et al. (1980)
Mytilus edulis	Cd 0.5 mg/l 14 days	Soft part	1 (MT-10)	Acetone precipitation DEAE–cellulose	Frankenne et al. (1980)
Mytilus edulis	Cd 0.1 mg/l 3–4 months	Soft part	4 (MT-20) 4 (MT-10)	DEAE–cellulose	Frazier et al. (1985)
Mytilus edulis	Hg 0.005 mg/l 28 days	Gills	3 (MT-20) 6 (MT-20)	DEAE–cellulose HPLC	Roesijadi (1986)
Mytilus edulis	Cd 0.2 mg/l 3–4 months	Soft part	5 (MT-20) 4 (MT-10)	DEAE–cellulose	Mackay et al. (1993)
Mytilus galloprovincialis	Cd 0.1 mg/l 3 months	Muscle	3 (MT-20)	DEAE–cellulose	Carpenè et al. (1983)
Mytilus galloprovincialis	Cu 0.04 mg/l	Soft part	1 (MT-10)	Acetone precipitation DEAE–cellulose	Viarengo et al. (1984)
Mytilus galloprovincialis	Cd 0.2 mg/l 20 days	Digestive gland, mantle	2 (MT-10–20) 1 (MT-10–20)	DEAE-Sephadex A-25	Pavičić et al. (1991)
Crassostrea virginica	Cd 0.2 mg/l 21 days	Soft part	2 (MT-10)	Acetone precipitation HPLC	Roesijadi et al. (1989)
Scapharca inaequivalvis	Cd 0.5 mg/l 28 days	Digestive gland	2 (MT-10)	DEAE–cellulose	Serra et al. (1995)
Dreissena polymorpha	Cd 0.2 mg/l 14 days	Soft part	2 (MT-10)	Acetone precipitation DEAE–cellulose	High et al. (1997)

dimeric forms has not been fully explained. We may speculate upon a greater mobility of metal bound bridging prior to the oxidation, particularly under certain physiological conditions during an increasing demand for metal ions (growth, reproduction, development) or under certain stressful conditions. Recently, in some invertebrate and vertebrate species, it has been found that the oxidative status of the cell may affect the mobility of Zn and Cu ions from MT by means of glutathione presumably based upon a disulphide–MT type of interaction (Maret 1994; Maret and Vallee 1998).

The present study on Cd-exposed *M. galloprovincialis* confirms a larger participation of dimeric components characterized by a higher metal-complexing capacity than presumably monomeric MTs, which are predominantly presented in control organisms (Fig. 1). The data reported in the literature are in accordance with this concept. For example, in natural populations of environmentally exposed *M. edulis*, only monomeric MT components (molecular mass 13,000) were detected in gills and viscera (Talbot and Magee 1978). Similarly, in the American oyster, *Crassostrea virginica*, the monomeric acetylated form of MT (molecular mass 10,000) was predominant in naturally-occurring organisms and in those experimentally exposed to the low Cd level (Roesijadi 1994). In Cd-exposed *M. edulis* (George et al. 1979; Roesijadi and Hall 1981; Köhler and Riisgard 1982; Frazier et al. 1985; Bebianno and Langston 1991; Mackay et al. 1993), the prevailing part of the total Cd-MT occurred in dimeric form. The proposed concept on differential Cd-mediated expression of specific MT forms was supported by evidence at the gene-expression level (Baršyte et al. 1999; Lemoine et al. 2000). The first reported cloning of *M. edulis* metallothionein cDNAs from the digestive gland followed by Northern blot analysis reveals that MT-20 isoforms, presumably produced by gene duplication events in *Mytilus* lineage, may be considered as a primarily inducible metallothionein not highly expressed in mussels under basal conditions where MT-10 isoforms were more prevalent.

A survey of the literature on the isolation of MT isoforms in different tissues of the genus *Mytilus*, as presented in Table 2, does not include wild mussels. According to our knowledge, the metal composition (Zn, Cu and Cd) of charge-distinct MT components isolated from naturally-occurring mussels (resolved by DEAE-chromatography) has not yet been reported. Our results show that Zn and Cu are the predominant metals associated with MT components of naturally-occurring mussels. This is consistent with the fact that the primary function of MT is believed to be their involvement in intracellular Zn and Cu homeostasis (Brady 1982). The presence of a low Cd level associated with these constitutive naturally-occurring MTs was also shown in our study. This is in agreement with some earlier observations (Overnell and Trewhella 1979; Frazier and George 1983), indicating that numerous marine invertebrates may accumulate a considerable concentration of cadmium even when growing in apparently unpolluted areas. A great similarity was observed upon metal distribution at the respective elution positions between comparative DEAE-profiles of control and Cd-exposed mussels (Fig. 5). The observed coincidence of metal-containing peaks may suggest that the resolved charge-distinct forms are expressed either at

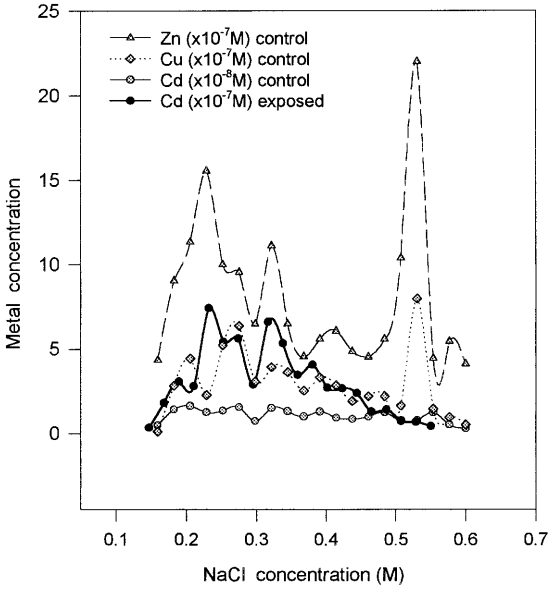

Fig. 5 Comparison of the metal distributions in DEAE -Sephadex A-25 MT-20 elution profiles between Cd-exposed and control mussels

basal (by Zn, Cu, Cd) or at an inducible Cd exposure level.

An interesting observation was made on the dimeric MT-20-I form eluted at 0.23 M NaCl (Fig. 5). This MT form contained a considerable level of Zn but no Cd in control mussels, and it was the most pronounced dimeric Cd-binding form in Cd-exposed mussels. Consequently, this individual MT-20-I form may be a more indicative biomarker of Cd exposure than the total MT content in the digestive gland. The specificity of its expression at a higher metal exposure level should be further evaluated by studying the inducible potentials of other metal ions upon de novo MT synthesis.

Most studies on MT isoforms in mussels involved their isolation from the pooled tissue from a larger number of individuals. Consequently, there is a possibility that differentially expressed MT alloforms occurring in different individuals could contribute to the total number of isolated MT forms from the composite sample as previously suggested by Mackay et al. (1993). Therefore, we separated Cd-MTs from samples derived from a single mussel in order to compare the resolved Cd maxima at the respective chromatographic profiles of the composite and single-specimen samples. According to our results, the single-mussel and the composite samples of digestive gland displayed a similar pattern of Cd distribution on the respective DEAE Sephadex A-25 profiles. This might suggest that the presence of multiple MT forms in a composite sample probably may not be attributed to polymorphism within the mussel population. Of course, further work and more sophisticated analytical methods are needed to substantiate such a statement.

The results obtained in the present study confirm the existence of the highly expressed MT multiplicity in the genus *Mytilus*, a phenomenon which has already been reported in the related species *M. edulis* (George et al. 1979; Frazier et al. 1985; Mackay et al. 1993). An apparently smaller number of MT forms in *M. gallprovincialis* reported in some previous publications (Viarengo et al. 1984; Pavičić et al. 1991) could be probably ascribed to methodological differences. Although the effect of different treatments on the separation of MT isoforms has not been systematically studied, by surveying the literature data from Table 2, it can be seen that the use of the organic solvent for the preparation of the MT-enriched sample resulted in an isolation of MT monomers, as well as in a smaller number of separated isoforms. This observation is in accordance with our preliminary results, suggesting that the ethanol fractionation, besides eliminating a part of the MT-20 component, may also contribute to a modification of specific isoforms.

In conclusion, by comparing the metal distribution in the respective chromatographic profiles, our results clearly indicate a considerable difference in the proportion of monomeric and dimeric MT forms between control and Cd-exposed mussels. Dimeric MT predominantly occurred in experimentally Cd-exposed organisms, suggesting that the dimeric component may be considered as a primarily inducible metallothionein. This finding may have practical implications for MT usage in biomonitoring. The higher proportion of dimeric MT forms, which appears to be transcriptionally induced by metal ions (Baršyte et al. 1999), may indicate the metal exposure of mussel field populations.

Our study confirms the multiplicity of MT forms in another species of the genus *Mytilus*, the Mediterranean mussel, *M. galloprovincialis*. Additional verification is needed in order to confirm that the observed multiple charge-distinct components could be ascribed to MT isoforms. According to our results, the occurrence of Cd-MTs in the digestive gland of single specimens is comparable to that of the sample composed of a larger number of mussels, suggesting that the high multiplicity of MT forms observed in composite samples is probably not be attributable to MT polymorphism within the mussel population.

Acknowledgements The financial support of the Ministry of Science and Technology of the Republic of Croatia for the project, No. 00981511, is gratefully acknowledged. The experiments were carried out according to the current laws of the Republic of Croatia. The paper is part of the MSc thesis of Dušica Ivanković.

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