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Quantitative determination of metallothionein-like proteins in mussels. Methodological approach and field evaluation

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Abstract. Electrochemical quantitation of metallothionein-like proteins (MLP) in mussels was based on the determination of their constituent cysteinyl residues according to Brdička's catalytic reaction. Calibration was performed by an internal MLP standard isolated from the digestive gland of *Mytilus galloprovincialis* for which protein concentration had been estimated by Bradford's spectrophotometric method. For that purpose three metal-binding proteins [MLP-I, MLP-II and Cu-BP (binding protein)] were separated by DEAE-Sephadex A-25 chromatography from the digestive gland of mussels previously exposed to Cd. The most negatively charged MLP-II fraction was characterized by the fact that it contained the largest amount of both total metal and sulphydryl (–SH) content per mass of protein, although this was approximately two times lower than the –SH level of commercially available MT from rabbit liver. Exposure of mussels to a relatively low level of cadmium (0.2 μg $Cd\,l^{-1}$) added into the seawater either by itself or as a mixture with other metals (2 μg $Cu\,l^{-1}$ and 1.6 μg $Pb\,l^{-1}$) resulted in a measurable level of MLP induction within 14 d in comparison to the control specimens. The effect of the metal mixture on MLP synthesis appears to be less than additive, suggesting a competitive interaction between metal ions for uptake and binding sites as well as differing potentials for MLP induction. Variations in the MLP content observed in the digestive gland of mussels seasonally collected from the same location are in the range $2.1 \pm 0.4\,mg\,g^{-1}$ on a wet weight basis. The methodological and conceptual aspects of the application of MLP induction in the *Mytilus* sp. as a biomarker in seawater trace metal monitoring are critically evaluated.

Introduction

Considerable interest in various aspects of the study of metallothionein (MT) since it was first isolated and characterized by Valle and co-workers in 1957 (Margoshes and Vallee 1957, Kägi and Vallee 1960) is consistent with its proposed key role in trace metal metabolism and detoxication mechanisms.

In spite of this, only a limited number of published papers deal with the quantitative aspect of MT and the determination of related proteins, especially those which have already been identified in the tissues of marine invertebrates (Olafson and Sim 1979, Lobel and Payne 1984, Roesijadi and Morris 1988, Bebianno and Langston 1991) and which differ to a certain extent from the fully characterized mammalian forms (Roesijadi 1981, Stone and Overnell 1985).

A critical and comprehensive survey of different methods for the quantitation of MTs based on their various physico-chemical properties appeared a few years ago (Cherian 1988).

Several modifications of a metal-saturation assay based on the affinity of MT and MT-like proteins for mercury (Pietrowski et al. 1973), cadmium (Chen and Ganther 1975) and silver (Scheuhammer and Cherian 1986) were widely exploited for the tissue quantitation of a relatively higher, induced level of mammalian MT (Onosaka et al. 1978). However, this method is not sensitive enough to measure the considerably lower MT levels in biological fluids as well as in control, metal-unexposed organisms.

Assuming greater diversity of the metallothionein-like protein (MLP) forms in mussels with respect to molecular mass and aminoacid composition (Frazier et al. 1985) in comparison to the close homology of well-defined mammalian MTs, even those originating from different sources, possible error may be expected if the metal-saturation method for the determination of MLP content in tissues is applied to *Mytilus* sp., or to organisms for which a molecular structure and precise number of metal-binding sites have not yet been fully determined. It has already been reported that the electrochemical method, based on Brdička's catalytic reaction (1933), is applicable for the detection of SH-containing compounds including MT and similar proteins in some species of marine invertebrates (Paleček and Pechan 1971, Olafson and Sim 1979,

Thompson and Cosson 1984, Raspor et al. 1987, Bebianno and Langston 1991). Assuming a larger molar proportion of cysteine residues in mammalian MT, our intention is to show that utilizing an internal MLP calibration standard isolated from the digestive gland of *M. galoprovincialis* instead of commercially available MT from rabbit liver may be more advantageous because this prevents an underestimation of MLP content.

The digestive gland was selected from among other tissues because it has the greatest capacity for MLP induction and an ability to reflect different concentration levels and exposure durations to cadmium experimentally added into seawater (Pavičić et al. 1987). Furthermore, by the applying the proposed method to the experimentally exposed and control mussels, the reliability of the trace metals control concept using MLP as a pollution indicator was been considered and critically evaluated.

Materials and methods

Mussels

Adult mussels with a mean shell length of 6 to 9 cm were collected from a mussel farm in the Lim Channel (western Istrian coast, North Adriatic).

Two experiments were performed on mussels previously exposed to different Cd levels: (1) mussels subjected to a high Cd level (200 μg Cd l^{-1}; for 20 d) and, (2) mussels exposed to a low Cd level (0.2 μg Cd l^{-1}; for 14 d), added as $CdCl_2 \times H_2O$ into the seawater using an open continuous-flow system. Simultaneously, in addition to a low-Cd concentration, another group of mussels was exposed to the "metal mixture" (0.2 μg Cd l^{-1} + 2 μg Cu l^{-1} + 2 μg Cu l^{-1} + 1.6 μg Pb l^{-1}) for the same exposure duration. Control, unexposed organisms were kept simultaneously in separate basins supplied with running seawater (no metals added).

Mussels used for monitoring the MLP level were collected every 3 mo from the same location in the Lim Channel (north Adriatic Sea). Composite samples of the digestive gland (10 to 15 specimens) were kept frozen at −20 °C until further treatment.

Isolation of MLP

The soluble phase of the previously homogenized digestive gland tissue was extracted by 0.02 *M* Tris-HCl buffer, pH 8.6 (3 ml g^{-1} tissue) containing dithiotreitol (1 m*M* DTT) and protease inhibitors (17 mg l^{-1} PMSF and 3 mg l^{-1} leupeptin).

The preliminary treatment of the sample, based on modified methods of Olafson et al. (1979) and Viarengo et al. (1980, 1984), has been described in detail elsewhere (Pavičić et al. 1985, 1989, Raspor et al. 1987).

Those samples, representing a crude homogenate (which were not later subjected to fractionation), were heat-treated at 70 °C for 10 min prior to the separation of the soluble phase by centrifugation (27 000 × *g*, 4 °C, 1 h) in order to precipitate interfering-SH-containing high-molecular mass proteins.

Particle-free supernatant, obtained by filtration through the membrane filter (0.2 μm) was further subjected to conventional liquid chromatography techniques. The initial separation step was gel-filtration using a Sephadex G-75 column (2.4 × 70 cm). The sample was eluted by 0.02 *M* Tris-HCl, pH 8.6, containing 0.5 m*M* DTT. When further purification was required, pooled chromatographic fractions containing Cd and absorbance at 250 nm maxima were applied to an DEAE-Sephadex A-25 column (1.1 × 33 cm) previously equilibrated by 0.02 Tris-HCl buffer, pH 8.6, containing 0.5 m*M* DTT, and eluted by an increasing concentration gradient of NaCl (0.05 to 0.4 *M*).

Analytical methods

Ultraviolet absorbance at 250 and 280 nm was routinely measured in chromatographic fractions using an UV/VIS Varian DMS 80 spectrophotometer.

Metal concentrations in chromatographic fractions were determined either by AAS (atomic absorption spectrophotometry), graphyte furnace (Pb) and flame mode (Zn), or electromchemically (Cd, Cu, Zn) using differential pulse anodic stripping voltammetry (DPASV) on the hanging mercury drop electrode. For electrochemical determination of metal concentrations a standard addition method was applied. Reference material was not utilized.

The polarographic method for determination of sulphydryl-containing proteins according to Brdička's (1933) reaction was applied in a manner described elsewhere (Raspor et al. 1987, 1991). The catalytic current of hydrogen evolution is measured at the potential of −1.6 V in a solution of 6×10^{-4} mol $Co(NH_3)_6Cl_3$ l^{-1} buffered to pH 9.5 with 1 mol l^{-1} ($NH_4OH + NH_4Cl$), using a DP polarographic mode.

Total protein concentration was determined according to Bradford's (1976) spectrophotometric method using Coomassie Brilliant Blue reagent. Commercially purified MT from rabbit liver (fractions I + II) was utilized as the calibration standard.

Results

Preparation of calibration standard

The isolation and partial characterization of the MLP fraction from the digestive gland of *Mytilus galloprovincialis* has been provided so that it can be applied as an internal calibration standard for quantitative determination of MLP content in mussels. For this purpose organisms exposed to the higher cadmium level (200 μg Cd l^{-1}; 20 d) were used. Following the gel-filtration step, pooled Cd-containing fractions, also characterized by the Cd-merkaptide bond (A_{250}) in the middle of a Sephadex G-75 elution profile (not presented here), were subjected to a DEAE-Sephadex A-25 column. Distributions of Cd, Cu and Zn shown in elution profile in Fig. 1 illustrate the presence of three metal-binding proteins designated as MLP-I, MLP-II and Cu-BP (binding proteins), with different proportions of associated metals. In addition to their chromatographic behaviour, partial characterization of isolation protein, presented in Table 1, clearly shows that two of them, MLP-I and MLP-II, possess general characteristics of the MT class owing to their high metal and sulfhydryl content and specific spectroscopic properties (A_{250}:$A_{280} > 1$).

The identity of the third fraction (CU-BP) was questionable because of its markedly lower metal and sulfhydryl content per mass of protein compared to MLP-I and MLP-II. Based on its spectroscopic and chromatographic properties as well as on its relatively high total protein content, this fraction, which possibly contains an oxidized MLP form due to the presence of predominantly bound copper (Suzuki and Maitani 1981), might be considered heterogenous (Viarengo et al. 1984).

It is interesting to note that each of the isolated protein fractions contains a large amount of copper although the mussels had been exposed to cadmium.

The calibration lines illustrated in Fig. 2 show spectrophotometric (A) and polarographic (B) determinations of protein content in some of the commercially pre-

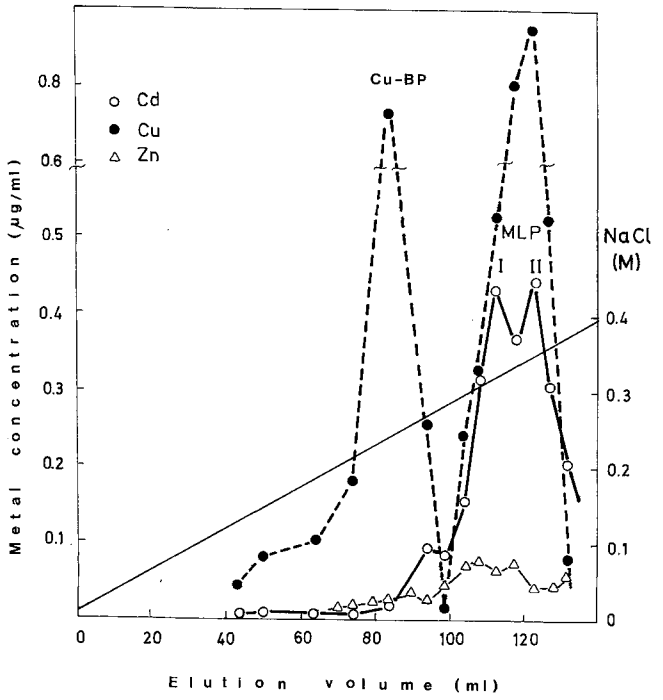

Fig. 1. *Mytilus galloprovincialis.* Metal distribution in DEAE-Sephadex A-25 profiles of digestive gland of mussels exposed to elevated cadmium concentrations in seawater (200 μg l^{-1}; 20 d). By application of a liner concentration gradient of NaCl (0.01 to 0.4 *M*) three protein fraction were resolved: Cu-BP (binding protein), MLP (metallothionein-like protein)-I and MLP-II

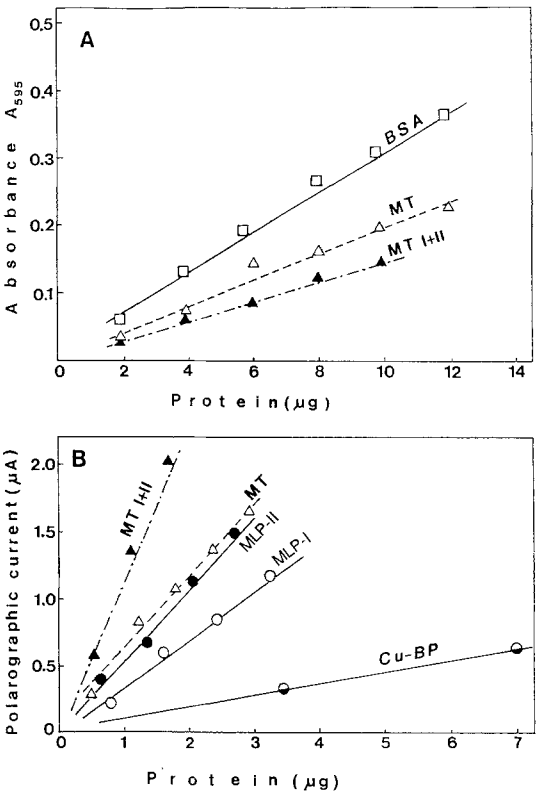

Fig. 2. *Mytilus galloprovincialis.* Quantitative determination of protein by spectrophotometric and electrochemical methods. (A) Bradford's spectrophotometric method showing linear relationship between a mass of commercially available protein standards (BSA: bovine serum albumine; MT: horse kidney metallothionein, MT I+II: rabbit liver metallothionein) and absorbance at 595 nm. (B) Electrochemical method according to Brdička's reaction showing comparatively calibration lines of isolated protein fractions from digestive gland of mussels [MLP (metallothionein-like protein)-I, MLP-II and Cu-BP (binding protein)] as well as from commercially purified metallothioneins from horse kidney (MT) and from rabbit liver (MT I+II)

Table 1. *Mytilus galloprovincialis.* Partial characterization of metal-binding proteins isolated from digestive gland of *M. galloprovincialis* using DEAE-Sephadex A-25 column chromatography. The characteristic UV absorbance ratio (A250:A280), Cd, Zn and Cu content as well as polarographic current intensity (Pol. cur.) expressed per mass of total soluble protein. Sulphydryl per total metal ratio (–SH:TM) of isolated protein fractions estimated. Polarographic response of commercially available metallothionein (MTs) from mammalian sources also presented. MLP: metallothionein-like proteins; BP: binding protein

Isolated proteins	A_{250}: A_{280}	Cd (μg-at. mg^{-1})	Cu	Zn	Pol. cur. (mA mg^{-1})	–SH[a]:TM (molar)
MLP-I	1.5	0.24	0.52	0.061	0.32	0.79
MLP-II	1.3	0.29	1.08	0.046	0.53	0.83
Cu-BP	1.3	0.004	0.16	0.005	0.12	1.66
MT-I+II (rabbit liver; "Sigma")					1.17	
MT (horse kidney; "Sigma")					0.59	

[a] Determined as 0.43 mA μmol^{-1} –SH of rabbit liver MT-I+II

pared proteins from mammalian sources as well as in metal-binding fractions from the digestive gland of *Mytilus galloprovincialis* isolated by our procedure. The spectrophotometric method according to Bradford (1976) enables quantitation of a total protein content beyond a molar mass of 3 kDa. Obviously, albumine from a bovine serum (BSA) produced markedly higher absorbance at 595 nm than both forms of mammalian MTs (from rabbit liver and horse kidney) owing to the great similarity of their aminoacid composition and protein structure (Kägi et al. 1984). The results obtained suggest that commercially purified MT forms are more appropriate when applied as a calibration standard for the spectrophotometric quantitation of the MT and similar types of proteins than the commonly used BSA.

The markedly higher polarographic current intensity of the rabbit liver MT-I+II compared to that found in the MT from a horse kidney (Fig. 2 B) may be ascribed to the better purification of the former protein. The presence of contaminating proteins in the MT form of horse kidney also reveals its higher total protein content, spectrophotometrically determined according to Bradford's method (Fig. 2 A). Intensities of the polarographic response, indicating sulphydryl levels by two forms of mammalian MTs, were in inverse order compared to the total protein content of the respective mammalian MT forms, suggesting better purification of the rabbit liver than of the horse kidney MT.

Furthermore, MT-I + II from the rabbit liver also displayed markedly higher sulphydryl content in comparison with each of the isolated MLP fractions from the digestive gland of *Mytilus galloprovincialis*.

This is in good aggreement with previously published results on aminoacid analysis in several isoforms of mammalian MTs, in which cysteinyl residues amount to 33 mol % (Kägi et al. 1984), and in the MT-like proteins of *Mytilus edulis* and *M. galloprovincialis* it ranges between 11.6 and 26.6 mol % (Viarengo et al. 1984, Frazier et al. 1985).

Quantitative determination of MLP and associated metal content in low-level metal induced mussels

Summarized amounts of Cd, Cu, Zn and Pb in chromatographic fractions corresponding to the MLP range and the respective intensity of Brdička's polarographic current in two low-level metal-exposed groups of mussels, as well as in unexposed control specimens, are presented in Table 2. The distribution of metals in the Sephadex G-75 elution profiles of the control and of the two metal-exposed groups of mussels, denoted as "Cd-only" and "metal mixture", is illustrated in Fig. 3 A. It also shows (Fig. 3 B) a high linear correlation ($r = 0.998$) between the total metal content and the respective Brdička's polarographic signal summarized in the individual chromatographic fractions belonging to the MLP region a heat-treated soluble phase of a digestive gland homogenate. The total –SH and MLP contents were estimated using MT-I + II (rabbit liver) and MLP-II (*Mytilus galloprovincialis* digestive gland), respectively, as calibration standards.

The elution profiles presented give some insight into the inducibility and affinity of specific metals toward MLP but may also reflect competitive interactions for uptake and binding sites among metal ions as a consequence of Cd-coexposure with Cu and Pb. In light of some previously reported controversial results dealing with the inducibility of Pb and its affinity for binding on MTs and related proteins (Talbot and Magee 1978, Yoshikawa and Ohta 1982), it should be noted that a small, but well-defined Pb maximum appeared at the MLP position on the elution profile obtained from mussels exposed to the metal mixture.

A significant distinction observed between the amount of Cd bound to the MLP fraction of the control group and to each group of metal-exposed mussels was much more pronounced than the difference between their respective sulphydryl contents. However, the amount of Cd bound to the MLP region in the elution profile of a Cd-only exposed group in comparison with the control, unexposed mussels (16 × higher), exceeded that of the group subjected to the metal mixture (13 × higher).

The results obtained for metal content in individual chromatographic fractions belonging to the MLP region on Sephadex G-75 profiles of two differently exposed groups of mussels, presented in Table 2, show that amounts of Cu and Pb are comparable or even slightly higher in the "Cd-only" than in the "metal mixture" group. In contrast, the Zn content of mussels subjected to the metal mixture was several times lower than that found in mussels exposed exclusively to the elevated concentration of Cd. A more plausible explanation of that effect suggests possible competition between Cd and other metal ions for uptake sites via biological membranes rather than an exchange of loosely bound Zn with Cu or Cd at

Table 2. *Mytilus galloprovincialis*. Summarized amounts (ng) of Cd, Zn, Cu and Pb determined in chromatographic fractions corresponding to MLP (metallothionein-like proteins) maxima in Sephadex G-75 elution profiles of control and two groups of low-metal exposed mussels ("Cd-only" and "metal-mixture"). Amounts of MLP and –SH estimated according to the polarographic current (Pol. cur.) measured using Brdička's reaction on SH-containing proteins. <DL: below detection limit; *: not measured; –SH:TM sulphydryl per total metal ratio

	Control	Cd-only	Metal-mixture
	(ng of metal bound to MLP)		
Metal			
Cd	12	198	153
Zn	<DL	1 534	173
Cu	4 004	8 264	7 917
Pb	*	82	67
Total metal (mol 10^{-6})	0.063	0.156	0.129
Pol. cur. (μA)	112	196	173
–SH (mol 10^{-6})[a]	0.259	0.453	0.400
–SH:TM (molar)	4.1	2.9	3.1
MLP (mg g^{-1})[b]	0.16	0.28	0.25

[a] Estimated taking into account 0.43 mA μmol^{-1} –SH
[b] Estimated on tissue wet wt taking into account 0.56 μA μg^{-1} MLP

Table 3. *Mytilus galloprovincialis*. Measurements of sulfhydryl-containing proteins according to Brdička's polarographic current (Pol. cur.) intensity (C) expressed per total protein content (P) in soluble phase of *M. galloprovincialis* digestive gland homogenate as well as their C:P ratio and total MLP (metallothionein-like protein) content per tissue wet weight. Mussels were sampled four times during their annual cycle from the same location (Lim Channel, North Adriatic). Mean shell lengths and their standard deviations also presented. SD: Standard deviation; CV: coefficient of variation

Sampling date	Shell length (mean ±SD)	Pol. cur. (μA ml^{-1}) C	Protein (mg ml^{-1}) P	Ratio (μA/mg) C:P	MLP (mg g^{-1} wet wt)
5 Apr 1990	8.2±0.5	4.8	0.56	8.6	2.6
14 Aug 1990	6.9±0.6	4.0	0.68	5.9	2.2
15 Nov 1990	7.4±0.6	3.7	0.26	14.2	2.0
1 Feb 1991	7.1±0.8	3.1	0.39	7.9	1.7
	Mean:	3.9	0.47	9.2	2.1
	±SD:	±0.7	±0.18	±3.6	±0.4
	CV (%):	17.9	38.3	39.1	19.0

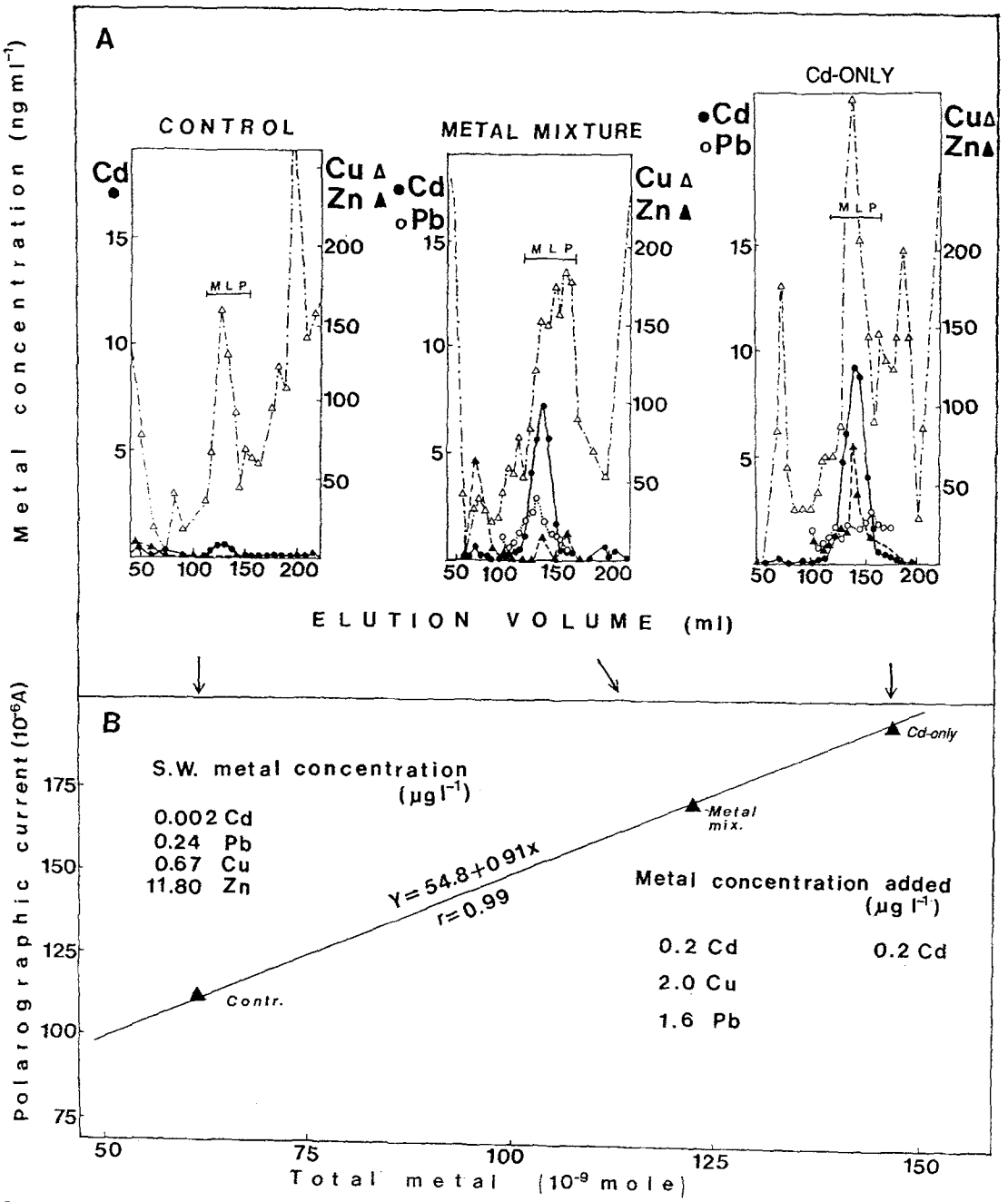

Fig. 3. *Mytilus galloprovincialis*. Mussels expossed to the low-metal concentration levels. (A) Metal distribution in Sephadex G-75 profiles obtained from soluble phase of digestive gland in control and two metal-exposed group of mussels (designated as "Cd only" and "metal mixture"). MLP: methallotionein-like protein. (B) Linear relationship between summarized total metal content (Cd + Cu + Zn + Pb; in 10^{-9} mol) and Brdička's polarographic current (10^{-6} A) determined in individual chromatographic fractions of respective MLP regions. Corresponding seawater (S.W.) metal concentrations also indicated. Contr.: control

MLP molecules, respective to their markedly higher values of stability constants. Consequently, an apparent release of Zn was not accompanied by an increased amount of Cu and Cd due to their higher affinity for binding sites at MLP molecules.

Seasonal monitoring of MLP levels in the digestive gland of mussels

The tissue MLP level was monitored in the digestive gland of mussels collected from the same location (the Lim Channel) at ca. 3-mo intervals during their annual reproductive cycle. The results based on Brdička's polarographic current (C) and the total protein content of the digestive gland homogenate extract (P) according to Bradford's assay and also in terms of their C:P ratio as well as MLP content tissue^{-1} wet wt are presented in Table 3. A coefficient of variation (CV) corresponding to the total soluble protein concentration recorded in different samples was considerably larger than that of the polarographic response, 38 and 18%, respectively. This disagreement among different samples, with respect to mea-

surements of the total soluble protein level, may be ascribed to seasonal variations rather than to an error in the extraction procedure because of the good agreement found between two replicates of the same sample. However, in spite of a considerable variation found among C/P data, tested by application of a variance *F*-ratio between two preparations of the same sample and four different samplings within an annual cycle, observed variations were considered statistically insignificant, possibly due to a small number of repetitive preparations. The total MLP concentration determined in the digestive gland of *Mytilus galloprovincialis* was 2.1 ± 0.4 mg MLP g^{-1} wet wt tissue.

Discussion

A proposed quantitative approach based on the combination of polarographic (Brdička's) and spectrophotometric (Bradford's) methods is more convenient for the determination of the MLP forms than quantitation by metal-saturation methods, particularly for those proteins for which molecular mass and metal-binding stoichiometry have not been precisely defined. Its applicability to the trace metal seawater control was examined by measuring the MLP content in the soluble phase of the tissue homogenate in the control, unexposed mussels as well as in individual chromatographic fractions of low-metal-level experimentally exposed organisms.

In the course of our previous studies (Pavičić et al. 1985, 1987) the digestive gland of *Mytilus galloprovincialis* was selected from among other tissues as the most appropriate for monitoring Cd and possibly other metals due to the fact that it showed the highest inducibility of MLP as well as the highest extraction efficiency of that protein from a tissue homogenate.

Furthermore, the present study was also been undertaken in order to find out whether inducible synthesis of the *Mytilus galloprovincialis* MLP by Cd itself, as well as in combination with other metals at relatively low concentration levels, could be used as a representative biomarker which indicates the exposure of mussels to elevated concentrations of trace metals in the surrounding water.

Advantages of polarographic determination

The polarographic method for the determination of sulphydryl-containing proteins according to Brdička's catalytic reaction has been proposed for the detection of MT and similar proteins because it has several advantages i.e., relative simplicity, high sensitivity and low detection levels, and it therefore may be suitable for large-scale screening in trace metals monitoring programmes (Pavičić et al. 1987, Raspor et al. 1987). A sample representing the soluble phase of a tissue homogenate, considered to be a postlysosomal fraction, could be directly analysed without being subjected to time-consuming, conventional liquid chromatography.

It should also be mentioned that some factors which may influence MLP determination by means of metal-saturation methods, especially the presence of interfering metals (Lobel and Payne 1987) as well as those factors generating inter-/intramolecular oxidation, do not affect the results of polarographic determination according to Brdička's reaction.

In a search for the origin of the high copper content associated with metal-binding proteins in the soluble phase of the high-Cd-exposed mussels, it has recently been determined that the level of copper bound to MLP fractions resolved on a DEAE-cellulose column was much lower than cadmium if the bicarbonate eluant buffer (NH_4HCO_3; pH 8.6) was used for the generation of the concentration gradient instead of Tris-HCl/NaCl as applied in the present study. Within this chromatographic run, the Cu-BP component was not isolated. This may suggest that the predominance of copper among the three metal-binding proteins isolated by DEAE-Sephadex A-25 in the present study was probably caused by exchange-reactions between Cu present as a contaminant in the eluant buffer and by less firmly bound Zn and Cd ions at the MLP molecule. Evidently, in spite of various proportions of metal ions bound to the isolated MLP-II components, the polarographic responses according to Brdička's reaction in two repetitive purifications of the same sample were practically of the same level (0.53 and 0.56 $\mu A\ \mu g^{-1}$ protein). Observed alterations in the metal composition of MLPs influenced by different conditions within ion-exchange chromatography demonstrate the advantages of polarographic quantitation of total MLP content which is not dependent upon the amount and metal species bound, as reported previously by Olafson and Sim (1979).

Furthermore, as has been previously shown, oxidative conditions accompanied by the reduction of divalent to the univalent copper form, bound exclusively to MT (Weser and Rupp 1979), may cause the formation of –SS– linkages at the expense of the cysteine residues. Such a reduction in the metal-binding capacity of MLP molecules (Suzuki and Maitani 1981, Tempelton and Cherian 1984) might result in an underestimation of the MT level based on metal-saturation techniques. In contrast, if oxidative formation of either intra- or intermolecular disulphide bonds should occur, it would not influence the intensity of the polarographic current. One should keep in mind that, in general, all active thiols will also produce catalytic current when present as the oxydized –SS– bonds (Müller 1963, Olafson and Sim 1979). This means that if intra-/intermolecular oxidation occurs during the processing of the sample it will not affect electrochemical quantitation of the MT and similar proteins according to Brdička's reaction.

With respect to the reliability of a trace metals monitoring programme utilizing the MLP of *Mytilus* sp. as a potential indicator of marine pollution, several problems have been recognized. They could be classified into two main aspects: methodological and conceptual. These aspects have been encountered during both our experimental work and the interpretation of the results obtained.

Methodological problems

The homogenization of a sample is the most critical step prior to analysis. Unequal homogenization of the samples may produce different extraction efficiencies which could contribute to the high dispersion of the results expressing the MLP content on a wet weight basis. It has already been reported elsewhere (Pavičić et al. 1985) that cadmium extraction efficiency in the soluble phase varies significantly in different tissues of *Mytilus galloprovincialis*. In order to control an extraction step, repeated homogenization of the same sample is required and determination of metal concentrations in the crude homogenate as well as in its soluble phase is recommended.

The presence of interfering high-molecular weight (HMW), SH-containing proteins may introduce a significant error, particularly in low-level metal-exposed and control samples. Our results show that a significant reduction of HMW maxima occurs when thermal treatment is applied. Furthermore, the addition of reductive agents (2-Me or DTT) in order to prevent MLP polymerization via an intermolecular –SS– linkage markedly improves resolution between the HMW and MLP fractions by means of a subsequent gel-filtration step which contributes to the more efficient purification of MLP.

On the other hand, the addition of reductive agents during the processing of the sample at the concentration level applied in the present study had no measurable effect on the determination of sulphydryl levels according to Brdička's reaction at the working potential used in our study (-1.47 V). This is also in accordance with the general statement of Olafson and Sim (1979) that the presence of tiolic compounds of low molecular mass does not contribute substantially to the polarographic signal at the same potential as cysteine-containing proteins.

The problem associated with the application of a proper calibration standard for quantitative determination of MT and similar proteins from invertebrate species has been recognized in several papers (Thompson and Cosson 1984, Wong and Rainbow 1985) assuming specific characteristics of non-mammalian MTs, particularly with regard to not yet precisely defined molecular mass and metal-binding stoichiometry.

Bearing in mind that MLP from mussels contain a significantly lower molar proportion of cysteinyl residues (Viarengo et al. 1984, Frazier et al. 1985, Roesijadi 1986), the quantitative method applied in the present study, based on the response of thiolic protein, would require an appropriate calibration standard, preferably isolated from the tissues of *Mytilus sp.* The results presented here demonstrate that the application of the highly purified internal standard isolated from the digestive gland of *M. galloprovincialis* using DEAE-chromatography would be more reliable for electrochemical quantitation of MLPs than utilization of calibration standards from mammalian sources.

According to our results, the MLP-II component would be the most appropriate for calibration purposes due to its markedly larger metal-binding capacity as well as due to its tiolic content which was the highest among the three components isolated (Table 1). The high degree of purity of the MLP-II component has been proved by comparing two repetitive isolations of the same sample by DEAE-chromatography (Pavičić et al. 1991). The electrophoretic profile (SDS-PAGE) indicated homogeneity of the MLP-II fraction (mol mass 12.6 kDa). Brdička's catalytic current was of the same level as for the calibration standard proposed in the present study, 0.56 and 0.53 $\mu A\ \mu g^{-1}$, suggesting also the high degree of purity attained by the procedure described.

Alternatively, according to our measurements, electrochemical data should be multiplied by a factor of 1.7 if commercially produced MT from rabbit liver is routinely applied as a calibration standard. This value has been estimated as a ratio between the number of cysteinyl residues (given in parenthenses) reported for several mammalian MT forms (20; Kägi et al. 1984) and in MLP of *Mytilus edulis* (12), recently found by Mackay et al. (in preparation) as reported by George (1991).

The polarographic quantitation of the MLP content in a whole soft part of *Mytilus edulis* using rabbit liver MT as a calibration standard was reported for the first time (Bebianno and Langston 1991). As mentioned before, our results clearly indicate that the polarographic signal of a rabbit liver MT was significantly higher than that of mussels, which necessarily would contribute to an underestimation of their MLP content. The data reported for the digestive gland of the control, unexposed mussels (8 mg g^{-1}) expressed on a dry mass basis, are comparable to our results (2.1 ± 0.4 mg g^{-1}) if the conversion factor of five, with respect to a wet/dry mass ratio, is used. In comparison with quantitative data obtained by the application of the immunoassay method (Roesijadi and Morris 1988), the results obtained by polarographic quantiation are generally higher than those obtained by alternative methods, including the Cd-saturation technique reported by Onosaka and Cherian (1982).

The comparison between polarographic determinations of MLP in the samples of different degrees of purification carried out in the present study shows that direct measurements in the soluble phase of a thermally-treated tissue homogenate are approximately one order of magnitude higher than results obtained by summarizing the MLP values determined in individual chromatographic fractions belonging to the MLP region on the Sephadex G-75 profiles.

This may be partialy attributed to the interfering SH-containing proteins as documented by the presence of a measurable amount of tiolic HMW proteins in elution profiles, which has been also observed in the study of Bebianno and Langston (1991), in spite of the application of heat-treatment.

Conceptual problems

These problems originate from the not yet clarified biological role played by MT and related proteins involved in the transport and accumulation of metal ions in bivalvae molluscs. An application of the strictly additive approach using results based on a single-metal exposure under laboratory conditions may result in considerable

deviation when extrapolated to the complex situation of the marine environment, where trace metals mostly participate as a mixture of both essential and non-essential elements in various combinations and concentrations. So far, the inducibility of only a few metals (Cd, Cu, Hg, Zn, Pb) has been examined in mussels showing unequal potential toward induced synthesis of MLP, although these metals were not applied in the mixture at concentration levels as low as those used in the present study. Bearing in mind that competitive interactions of different metal ions added to seawater may occur at different organization levels, i.e., at the sites of metal uptake through an outer body membrane of an organism or some particular organ/tissue, such processes may alter the accumulation and redistribution of different metals among tissues and subcellular constituents, particularly due to competitive interaction among the binding sites on the MLP molecule. With regard to the different induction potentials of specific metal ions, such redistributions may produce an unpredictable response measured by the induced MLP levels when correlated with the trace metals composition of seawater.

Our results from a study dealing with the induction of the MLP using a metal mixture, which showed a slight reduction of the total MLP content in comparison to the effect of cadmium alone (Raspor and Pavičić 1991), may be explained by similar processes. Although the difference observed in the induced MLP level between Cd only and Cd applied in the mixture with other metals has not been found to be statistically significant, it may suggest competitive interaction between Cd/Zn or Cu/Cd at the binding sites of the MLP molecule and possibly at higher organization levels, as previously observed in some marine and freshwater species of bivalves (Jackim et al. 1977, Hemelraad et al. 1987). In contrast, co-accumulation of certain metal ions has been recorded in *Mytilus edulis*, *Macoma baltica* and *Crassostrea virginica* under exposure to the metal mixture (Frazier 1979, Kaitala 1988), while deviation from an additive model in *M. edulis* has not been found (Phillips 1976). Similar effects on metal redistribution were observed in the selected tissues of *M. edulis* following exposure to copper which had been affected by the seawater concentration of the metal added (Harrison et al. 1983).

It has been also suggested that the effect of biotic factors on the rate of growth at various coastal locations may seriously influence concentrations of metals accumulated in the soft part of *Mytilus galloprovincialis* (Martinčić et al. 1992). Furthermore, the fluctuation of the accumulated level of several metals within the reproductive cycle of *M. edulis* (Coimbra and Carraca 1990) has recently been reported. Considering the effects of various biotic factors on the fluctuation of the MLP content, these results should not be neglected. The effect of corticosteroid hormones upon induces synthesis of MT has been documented in mammals. In this connection, the presence of progesteron during redevelopmental stages of *M. edulis* has recently been recorded (Coimbra and Carraca 1990), suggesting possible fluctuations of the baseline MLP level within the annual reproductive cycle of mussels. In addition, a high requirement for essential metal ions, particularly zinc in rapidly growing tissues, was found to be accompanied by the increased synthesis of MT in prenatal stages of mammals (Brady 1982).

Our results showing higher MLP as well as higher total soluble proteins content during the spring-summer period than during the autumn-winter season are indicative for variations of the baseline MLP level in the range $\pm 19\%$, although the observed differences are beyond a statistical significance. At present, it is unclear whether or not the variations of constitutive the MLP level could be ascribed exclusively to biotic factors or possibly to biotic factors combined with abiotic factors, particularly those associated with the progressive decrease of the water temperature during the autumn-winter season which may generally alter biosynthetic processes. A better understanding of trace metals metabolism in mussels would contribute to a more realistic explanation and evaluation of interfering biogenic and possibly other factors involved in MLP induction and would help to provide a more conclusive evaluation with respect to the applicability of the proposed biomarker in seawater control.

Conclusions

The results obtained confirmed that Brdička's reaction for the determination of sulphydryl-containing proteins using DP polarography could serve as a routine method for large-scale screening of the MLP content in environmentally exposed populations of mussels. The baseline level of MLP determined in the digestive gland of mussels collected from the Lim Channel varies between 1.7 and 2.6 mg g^{-1} on a wet weight basis. The recorded MLP fluctuations within the annual reproductive cycle, although beyond statistical significance, may be ascribed to the joint effect of biotic and abiotic factors.

The combined effect on MLP induction in mussels of three metals (Cd + Cu + Pb) simultaneously added into the seawater, subjected to a relatively low concentration level compared to coastal pollution, is lower than that induced by a cadmium-only exposure. This effect may be attributed to the competitive interactions of metals for both uptake and MLP binding sites as well as to the different induction potentials of the metal in question on metallothionein synthesis.

In addition to certain methological improvements techniques for extracting and precipitating the interfering proteins prior to quantitative determination, an application of a proper internal calibration standard is recommended in order to avoid underestimation of the MLP content due to a significantly lower molar proportion of cysteine residues in comparison with MTs from mammalian sources. A comparison with a more specific, demanding, and expensive immunoassay method as well as the application of additional biological parameters might lead to a better understanding and control of MLP variations during the reproductive cycle of mussels.

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