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ADVANTAGES AND DISADVANTAGES OF VOLTAMMETRIC METHOD IN STUDYING CADMIUM-METALLOTHIONEIN INTERACTIONS

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Abstract - A sensitive and chemical species-selective technique of differential pulse anodic stripping voltammetry (DPASV) was applied in studying the cadmium-metallothionein (Cd-MT) interaction. The amperometric titrations of the purified MT20 and MT10 fractions, isolated by verified biochemical procedures from the digestive gland of cadmium-exposed mussels *Mytilus galloprovincialis*, with Cd^{2+} ions were performed in the buffered sodium chloride solution of 0.59 M ionic strength, pH 7.9 and 25°C. Applying the DPASV method at various cadmium to metallothionein ratio several groups of chemical species were recorded. The data on the available ligand concentration to complex cadmium ions (C_L), the apparent concentration stability constants (K') of the respective complexes and the reliability of the determined complexing parameters are discussed. In quantifying the Cd-MT interaction the interference of dithiotreitol (DTT), which is used as the reducing agent in isolation and purification of MTs, is documented.

Key words: Metallothionein, isoforms, dithiotreitol, cadmium binding, *Mytilus galloprovincialis*, voltammetry

Abbreviations: C_L: available ligand concentration for complexing cadmium; $[Cd_{ionic}]$: $[Cd^{2+}]_{aq} + [CdCl^+] + [CdCl_2]$; **DEAE**: diethylaminoethyl; **DPASV**: differential pulse anodic stripping voltammetry; **DTT**: dithiotreitol; **HMDE**: hanging mercury drop electrode; **K'**: apparent concentration stability constant; **MT**: metallothionein; **PMSF**: phenylmethylsulfonylfluoride; **Tris**: tris(hydroxymethyl)aminomethane

INTRODUCTION

Metallothionein (MT) is a low molecular mass, cysteine-rich metal binding protein discovered some 40 years ago. It has been shown that MT is widely distributed, occurring in prokaryotes, protists, fungi, plants and animals (Hamer, 1986). The widespread occurrence of MT in the aquatic species is well documented (Roesijadi, 1992). Metallothioneins (MTs) are known to bind metals such as Zn and Cu (which are essential for cell growth and development) and Cd and Hg (which are toxic) giving a clue about their role in metal toxicity regulation and homeostasis (Viarengo, 1989). Many fish and aquatic invertebrate species posses proteins that have features consistent with MTs. However, those from only a few species have been purified and characterized to the extent that has permitted analysis at the structural level. As a result, our understanding of the biochemistry of MTs from the aquatic species is limited (Roesijadi, 1992). The organs in which MTs are concentrated in aquatic animals are the liver (or equivalent organs in invertebrates). kidney, gills and intestines. In the aquatic invertebrates, including the *Mytilus* sp., most frequently studied are the digestive gland or hepatopancreas and gills, but not uncommon is to study the whole soft part of the molluscs and small invertebrates. It has been proposed that in biological systems MT has different roles; one of them is related to metal detoxification. From that point of view it is important to study and quantify the cadmium-metallothionein interactions at the concentration level close to the cellular one.

In general, there is a lack of data on complexation of Cd^{2+} ions by the purified MT isolated from the mussel *Mytilus galloprovincialis* being exposed to cadmium. Mussels are sessile and filter-feeding marine organisms which are widely distributed and used as indicators of metal pollution of the coastal seawater. For several molluscan species molecular mass variants on Sephadex, suggestive of monomeric and dimeric forms of about 10 kDa and 20 kDa, i.e. MT-10 and MT-20, respectively, have been reported. Two groups of isoforms MT-10 and MT-20 have been purified from the whole soft part of *Mytilus edulis* and sequenced by Mackay *et al.* (1993). The same authors confirmed that cadmium-binding protein, isolated from mussels belong to the class I metallothionein and exibits more similarity to the vertebrate MTs than to those of non-molluscan invertebrates. The authors Baršyte *et al.* (1999) have cloned and characterized several subtypes of both MT isoforms from *M. edulis* digestive gland in order to clarify how the MTs are evolving and to get an insight into the discrete functions of each genetic subtype of the protein. The authors have concluded that MT-20 isoform represents a primarily inducible MT not highly expressed under basal conditions.

The intention of our study is to evaluate the apparent stability constants of Cd-MT complex at biologically relevant concentration of cadmium and MT isolated with the reliable biochemical procedures from the *Mytilus galloprovincialis* digestive gland. Such data are missing in the literature. The intention is further on to bring out few critical remarks which might help in future studies in order to select the optimal experimental conditions for Cd-MT complex formation.

MATERIALS AND METHODS

Chemicals

All solutions for voltammetric analysis were prepared from Suprapure grade chemicals by Merck and ultrapure water for trace metal analysis by Water Pro PS system from Labconco (USA). As the supporting electrolyte for voltammetric measurements a 0.59 M NaCl solution of chloride content similar to the seawater, was used. The standard Cd^{2+} solution (1.000 ± 0.002 g l^{-1}) which contains $CdCl_2$, was prepared from Titrisol solution (Merck, Germany).

The studied cadmium-binding properties refer to the metallothionein fractions MT-20 and MT-10 isolated from the digestive gland of cadmium-exposed mussels, *Mytilus galloprovincialis*.

Isolation and purification of mussel MT

Adult specimens of *M. galloprovincialis* (5-7 cm, from the Limski Kanal, North Adriatic) were exposed for 14 days to 200 µg Cd dm^{-3} (added as $CdCl_2$) in a continuous flow-through seawater system (S=38 psu, 20°C). The composite sample of the digestive gland of cadmium-exposed *M. galloprovincialis* was homogenized in a biochemical mixture consiting of 0.020 M

Tris-HCl buffer (pH 8.6), the protease inhibitors (PMSF, leupeptine) and reducing agent (0.001 M DTT). For homogenization, the mass ratio of mussel tissue toward the biochemical mixture was 1: 3. Supernatant was separated by centrifugation during 40 min. at 30.000 g and 4°C. The supernatant was fractionated by gel filtration on a Sephadex G-75 column with an elution solution composed of 0.020 M Tris-HCl buffer pH 8.6 and 0.001 M DTT. Several parameters like:
- the content of cadmium;
- the content of mercapto groups according to Nielsen and Winge (1984)
- the absorbance at 254 nm (ascribed to the absorption of cadmium-sulfhydryl bond)
- the ratio of the absorbances at 254 and 280 nm,
support the fact that MT is eluted in the middle range of the elution profile. The component of MT dimer, denoted as MT-20, was obtained by pooling nine chromatographic fractions (Jurič, 1998). MT-20 component was additionally purified by anion-exchange chromatography on diethylaminoethyl (DEAE) Sephadex A-25 column (1.5 x 4.5 cm) and eluted with a linear concentration gradient of NaCl (0.02 M to 0.6 M) in 0.020 M Tris buffer (pH 7.4) and the flow rate of 0.26 ml min^{-1}. On the elution profile in fig. 1 seven maxima could be noticed (Jurič, 1998). For further electrochemical studies the purified fractions 14 and 18, abbreviated as MT-20(14) and MT-20(18), were selected because of their high cadmium content and the fact that MT-20(14) was assigned as the form with the highest inducibility by cadmium (Jurič, 1998).

The content of MTs in the elution fractions MT-20(14) and MT-20(18) was determined by the modified Brdička procedure (Brdička, 1933) which is a method of polarographic determination of sulfhydryl-containing proteins in ammonia-buffered cobalt(III) salt solution, applying differential pulse voltammetric mode (DPV) (Raspor and Pavičić, 1996). The basis for the quantification of proteins containing SH-residues (including MTs) is in the linear relationship between the height of the hydrogen catalytic wave and the protein concentration. The MT concentration present in the aliquots of MT-20(14) and MT-20(18) was determined from the height of the catalytic hydrogen wave and the slope of the calibration straight-line, obtained with a commercially available rabbit liver MT-(I+II), produced by Sigma (St. Louis, USA).

The total cadmium content in the purified MT-20(14) and MT-20(18) was determined by a standard addition method in a differential pulse anodic stripping mode (DPASV) at a hanging mercury drop electrode (HMDE) (Raspor *et al.*, 1998) in a solution acidified with HCl to pH<2. The results on the metallothionein, total cadmium concentration and their ratio

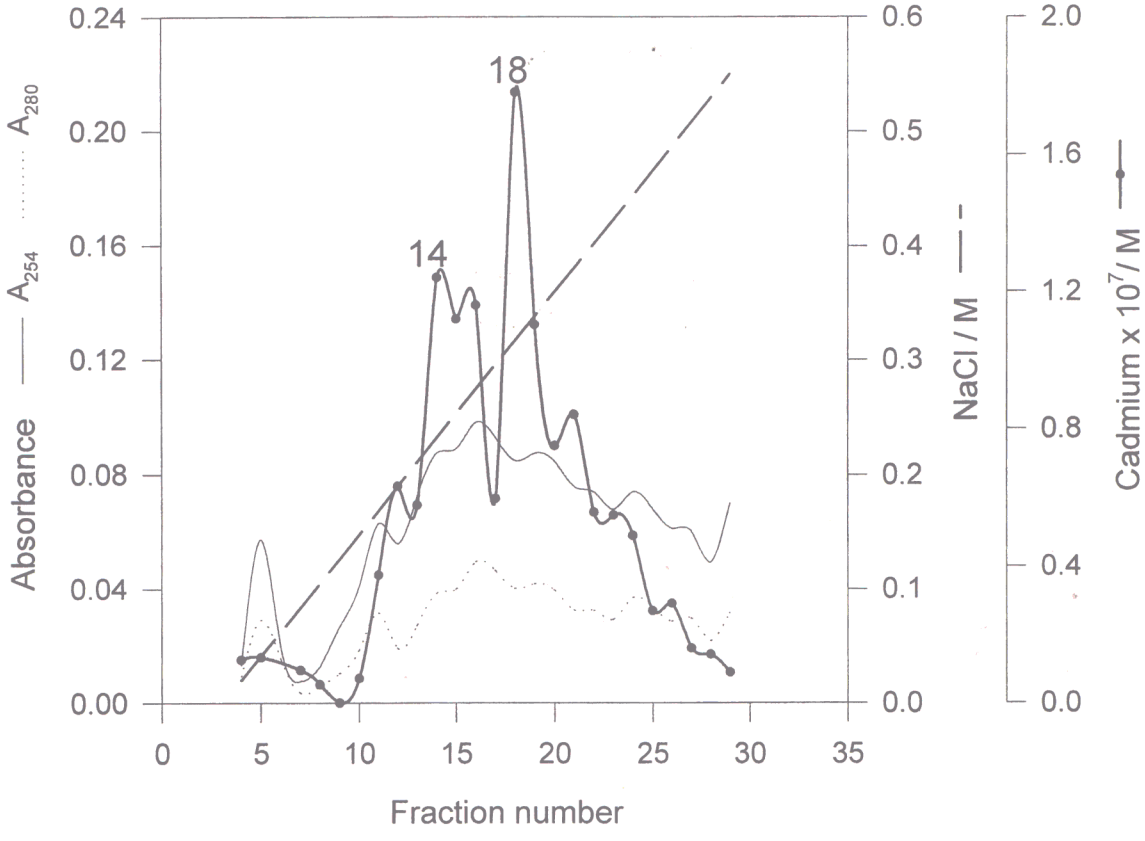

Fig. 1 *Elution profile of the pooled MT-20 components, isolated by gel filtration method, from the anionic exchange column on DEAE Sephadex A-25* (1.5 x 4.5 cm), *with the linear concentration of NaCl* (0.02 M to 0.6 M) *in 0.020 M Tris-HCl buffer* (pH 7.4) *at the flow-rate of 0.26 ml* min^{-1}. In the elution fractions the absorbances at 254 and 280 nm and the cadmium distribution are indicated.

Table 1 *The concentrations of MT and total cadmium (Cd_T) in two purified MT-20 fractions, isolated from the digestive gland of Mytilus galloprovincialis*

Chromatographic Fraction No.	10^7 [MT] / M *	10^7 $[Cd]_T$ / M *	Ratio $[Cd]_T$/[MT]
MT-20(14)	1.14 ± 0.12 (n=11)	1.14	1.0
(with DTT)	1.34 ± 0.31 (n=6)	1.35	1.0
MT-20(18)	0.99 ± 0.18 (n=12)	1.60	1.6
(with DTT)	1.26 ± 0.11 (n=9)	1.97	1.6

*Two independent measurements; The studied chromatographic fractions are indicated on the elution profile in fig. 1 and they contain beside MT DTT, too.

are summarized in table 1, and refer to the original, undiluted chromatographic fractions. For the selected MT-20 fractions we dispose only with the data on cadmium content and not on copper and zinc content.

Instruments

The system for gel-chromatography was from Pharmacia (Uppsala, Sweden), including the automatic fraction collector. Centrifugation was performed with the Sorval RC28S centrifuge by Du Pont (Wilmington, Delaware, USA). Spectrophotometric measurements at 254 and 280 nm were performed with a Cary 4 instrument (Varian, Australia). Voltammetric measurements were carried out with a μAutolab instrument (Eco Chemie, The Netherlands), The temperature was kept constant at 25.0 (± 0.5)°C with a laboratory water ciculator (Haake D8). The pH was measured with a Metrohm (Germany) Model E603 pH meter and a Metrohm combined pH electrode after proper calibration.

Amperometric titration with Cd^{2+}

The measurements in a differential pulse anodic stripping voltammetric mode (DPASV) were performed in a 25 ml Metrohm-type voltammetric cell at the working, HMDE Metrohm EA290 (Switzerland), in 0.59 M NaCl solution used as the supporting electrolyte. The reference electrode was an Ag | AgCl | $KCl_{sat.}$ electrode connected to the cell via a salt bridge filled with 0.59 M NaCl. The counter electrode was a platinum wire. Measurement parameters were set up for the DPASV mode with a deposition potential at -0.9 V versus a reference electrode, a deposition time of 120 sec., a resting time of 30 sec., a pulse amplitude of 0.025 V, a pulse duration of 0.057 sec., a scan rate of 0.005 V s^{-1} and a clock time of 0.5 sec. During the metal deposition step the solution was stirred with a Teflon coated magnetic bar at 800 rpm, to speed up the mass transfer of solution to the electrode. All measurements were performed at a constant temperature of (25.0 ± 0.5)°C. Before the voltammetric measurements the solution was deaerated for approximately 15 min. with a stream of extra pure nitrogen, which passed during the measurement over the surface of the measuring solution.

The aliquots of the chromatographic fractions MT-20(14) and MT-20(18) were amperometrically titrated in a DPASV mode, with the standard $CdCl_2$ solution. After the titrant addition, the solution was equilibrated by stirring and purging the nitrogen. The dilution factor of the original chromatographic fractions was 400, i.e. 20 ml of the electrolyte contained 50 μl of the respective chromatographic fraction.

RESULTS

In fig. 2 the characteristic groups of signals recorded during the amperometric titration of 50 μl of the chromatographic fraction MT-20(14) and MT-20(18) in the system of 0.59 M NaCl, borate buffer pH 7.9, are summarized. Before starting the titration with Cd^{2+} ions (Fig. 2, curve 0), observable is only the signal I at -0.58 V vs. the reference electrode, which corresponds to the uncomplexed ligand. The titration with the defined Cd^{2+} concentrations follows and the other groups of signals are recorded. During the titration signal I progressively decreases, while the signal denoted as II, recorded in the potential range from -0.69 to -0.72 V, progressively increases and corresponds to the signal of cadmium complexed with the ligand. When the signal of group II reaches the maximum height, the signal I completely disappears and a signal of the ionic forms of cadmium appears with the peak potential -0.61 V. This group of signals is denoted as III. The fourth group exists, too, with E_p at -0.40 to -0.44 V attributed to the oxidation of the mercury electrode in

the presence of thiol groups (Stankovich and Bard, 1977) complexed by cadmium (Mendieta *et al.*, 1995; Ruiz *et al.*, 1995). Signal IV increases in peak size with the addition of Cd^{2+} concentration, and attains the maximum at about twice the concentration of added $CdCl_2$ in order to attain the maximum peak size of signal II, which is related to the cadmium complexed by the ligand. As the titration with $CdCl_2$ begins (Fig. 2), the signal I of the uncomplexed ligand continuously decreases, while the signal II of the cadmium complexed by the ligand continuously increases up to a maximum value; then the signal III of the ionic forms of cadmium appears and starts to increase. In fig. 3 the peak currents of each group of signals versus the added $CdCl_2$ concentration, in the range from 2.5 x 10^{-9} M to 4.5 x 10^{-8} M, are presented. From that presentation the previously explained interrelation of four groups of signals is obvious. The voltammograms in groups II, III and IV derive from the electrode processes which involve cadmium (Nyberg and Zhou, 1995).

The peak currents of signals II (referring to $ICd_{complexed}$) and III (referring to ICd_{ionic}) are further on used for the evaluation of the available ligand concentration (C_L) for complexing Cd^{2+} ions and for the determination of the apparent stability constant (K'). The evaluation is done according to the modified Ružić procedure (Erk and Raspor, 1998, 1999) and is applicable to the inert type of complexes. Due to the fact that the signal of cadmium complexed by the ligand is separated from the signal of Cd_{ionic}, the Ružić procedure (1982) valid for the case when complexing occurs with one type of ligand or functional groups, was modified accordingly:

$$ICd_{ionic} / ICd_{complexed} = (1/K'C_L) + (1/C_L)\,[Cd_{ionic}] \quad (1)$$

When titrating 50 µl of MT-20(14) or MT-20(18), a straight-line relationship was obtained (Fig. 4), which justifies the complex formation with one type of ligand. The $[Cd_{ionic}]$ was determined from ICd_{ionic}

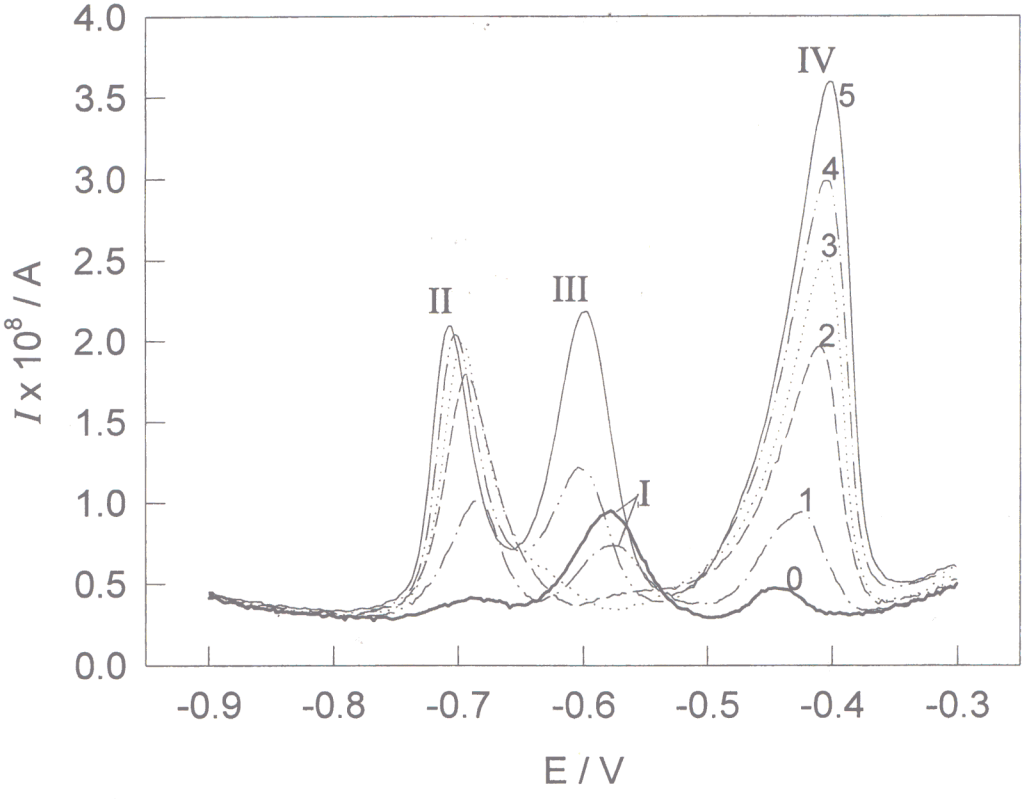

Fig. 2 *Current-potential curves in a DPASV mode in 0.59 M NaCl, borate buffer pH 7.9, containing 50 µl of the chromatographic fraction MT-20(14), curve 0; the titration with $CdCl_2$ was performed at 25°C in the concentration range from 2.5 x 10^{-9} M to 4.5 x 10^{-8} M.* Presented are the voltammograms at c($CdCl_2$), curve 1) 5 x 10^{-9} M; 2) 1.5 x 10^{-8} M; 3) 2.0 x 10^{-8} M; 4) 3.0 x 10^{-8} M; 5) 4.25 x 10^{-8} M.

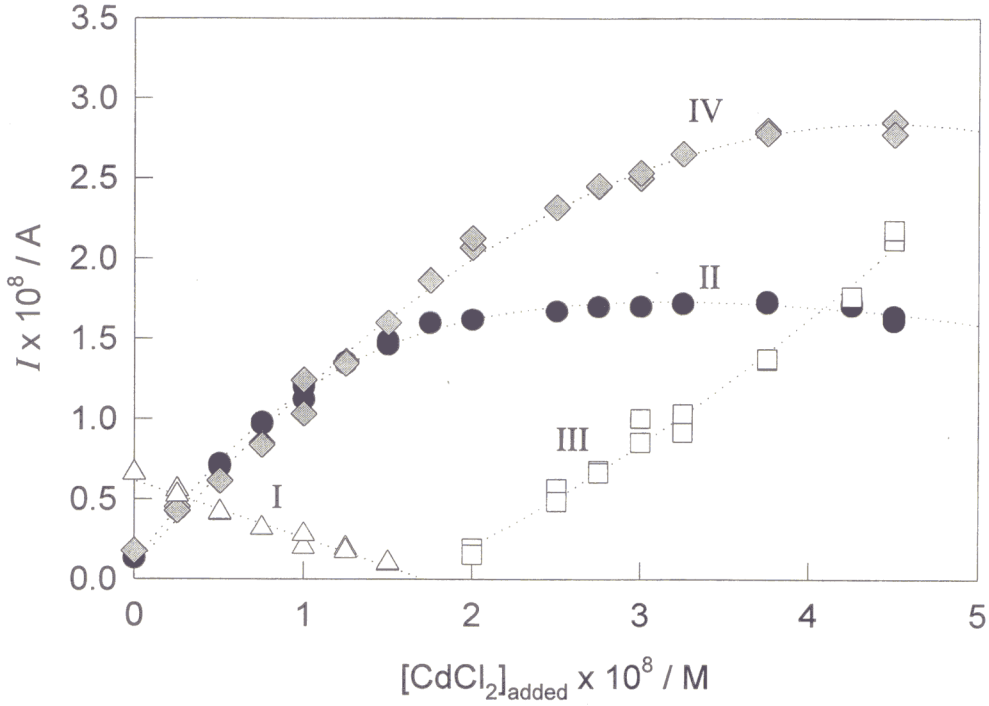

Fig. 3 *Peak currents of four groups of species indicated as I to IV in fig. 2, being recorded in a DPASV mode during the titration of 50 µl chromatographic fraction MT-20(14) with c($CdCl_2$) from 2.5 x 10^{-9} M to 4.5 x 10^{-8} M.* For each group of species, the peak potentials (E_p), expressed against the Ag/AgCl reference electrode, are the following: I) -0.58V; II) -0.68V; III) -0.61; IV) -0.44 to-0.40V.

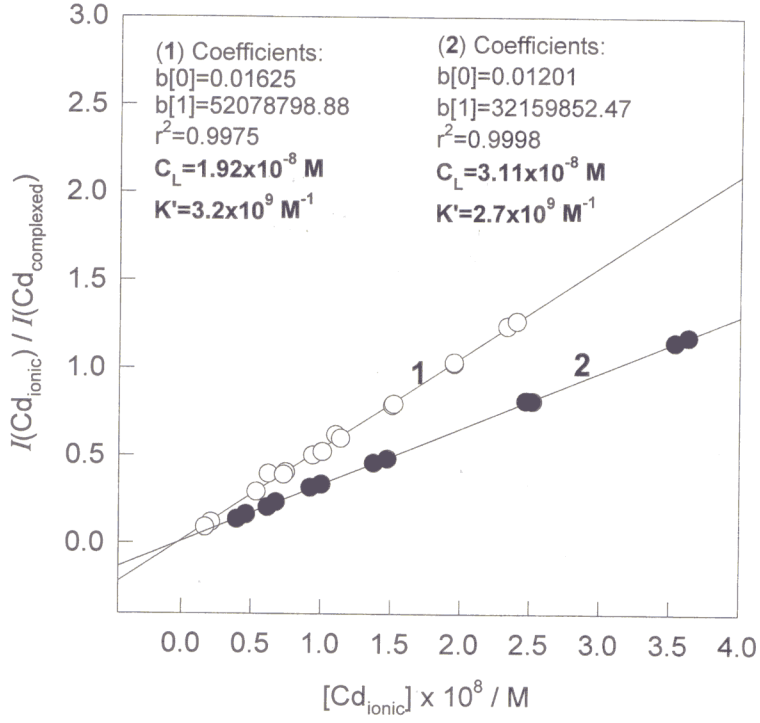

Fig. 4 *Plot of the ratio of the ionic cadmium peak current (ICd_{ionic}) to the peak current of cadmium complexed with the ligand ($ICd_{complexed}$) versus the ionic cadmium concentration.* The electrolyte composition and the measuring conditions are the same as for the results presented in figs. 2 and 3. The presented data refer to the titration with the defined c($CdCl_2$) of 50 µl of each chromatographic fraction: 1) MT-20(14) and 2) MT-20(18), which contain beside MT DTT, too.

and the slope of the calibration straight-line for cadmium which was recorded in the absence of ligand, under the same electrolyte, pH and temperature conditions. From the slope of the straight-line according to eq. 1 the available capacity of the ligand (C_L) for complexing Cd^{2+} ions can be determined while from the intercept ($1/K'C_L$) the apparent stability constant (K'). The C_L of 50 µl chromatographic fractions MT-20(14) and MT-20(18), diluted in 20 ml of the buffered supporting electrolyte, amounts to 1.92×10^{-8} M and 3.11×10^{-8} M, respectively (Table 2). If corrected for the dilution, the C_L of the original, undiluted chromatographic fractions MT-20(14) and MT-20(18) would amount to 7.68×10^{-6} M and 1.24×10^{-5} M, respectively. The K' values for MT-20(14) and MT-20(18), according to eq. 1 amount to 3.2×10^{9} M^{-1} and 2.7×10^{9} M^{-1}, respectively (Table 2). For the determined complexing parameters by means of the computor program (Pižeta and Branica, 1997) the confidence limits for 95% level of confidence were calculated and reported.

The results presented in figs. 2 to 4 refer to evaluation of the binding properties of the specific ligand in the pooled chromatographic component MT-20, isolated from the digestive gland of cadmium-exposed *Mytilus galloprovincialis*, and eluted from the anion-exchange Sephadex A-25 column. The fact that during the titration with $CdCl_2$ the separate signals of $Cd_{complexed}$ and Cd_{ionic} (II and III in Figs. 2 and 3) are observed indicate that the cadmium bound to ligand is an inert type of complex. Analyzing these results two facts were contradictory to our already reported results (Erk and Raspor, 1998, 1999):
the appearance of the signal I, at -0.58 V vs. the reference electrode, attributed to the uncomplexed, electroactive ligand;
C_L values of the original, undiluted chromatographic fractions (explained above) roughly two orders of magnitude higher than MT content estimated by an independent method (Table 1).

The possibility was considered that the purified chromatographic fractions contain beside MT an additional ligand, at higher concentration than MT, which complexes Cd^{2+} during the titration. The results presented in table 1 indicate that the unknown ligand does not interfere with the measurements of

Table 2 *Cadmium complexing capadity (C_L), the apparent stability constant (K') and their product determined in 0.59 M NaCl, pH 7.9 (borate buffer) at (25.0 ± 0.5)°C according to eq. 1.*

Chromatographic fraction No.	10^{8} [C_L] / M	10^{-9} K' / M^{-1}	Number of data sets	K' C_L
MT-20(14) (with DTT)	1.92 ± 0.14	3.20 ± 10.15	16	61
MT-20(18) (with DTT)	3.11 ± 0.07	2.68 ± 3.05	12	83
MT-10(V)*	1.13 ± 0.04	2.83 ± 3.18	8	32
(without DTT)	1.00 ± 0.04	3.46 ± 4.25	5	35

The confidence limits for 95% level of confidence, calculated according to Pižeta and Branica (1997) are reported too. Dilution of the original chromatographic fractions is 400. As indicated, single MT-20 fractions contain beside MT DTT, too, while the pooled MT-10(V) fraction contains MT only because DTT was beforehand removed.
*Two independent measurements

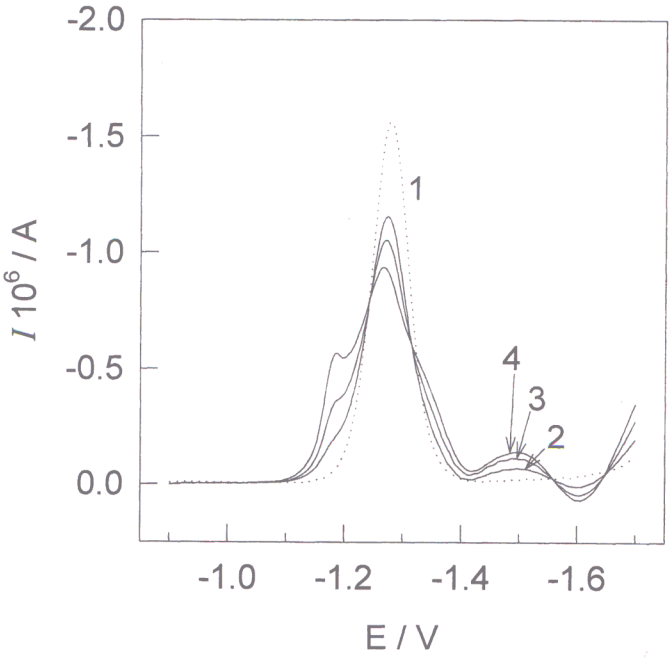

Fig. 5 *Modified Brdička method for the determination of MT content isolated from the digestive gland of cadmium-exposed mussels M. galloprovincialis.* Curve 1 represents the reduction of Co^{2+} to Co^{o} in the absence of MT. Curves 2, 3 and 4 were recorded after equal aliquots (10 µl each) of single MT-10(V-32) fraction, which contains MT and DTT, were added. MT content is evaluated from the height of the signal at -1.5 V, while the signal related to DTT is observed at -1.17 V.

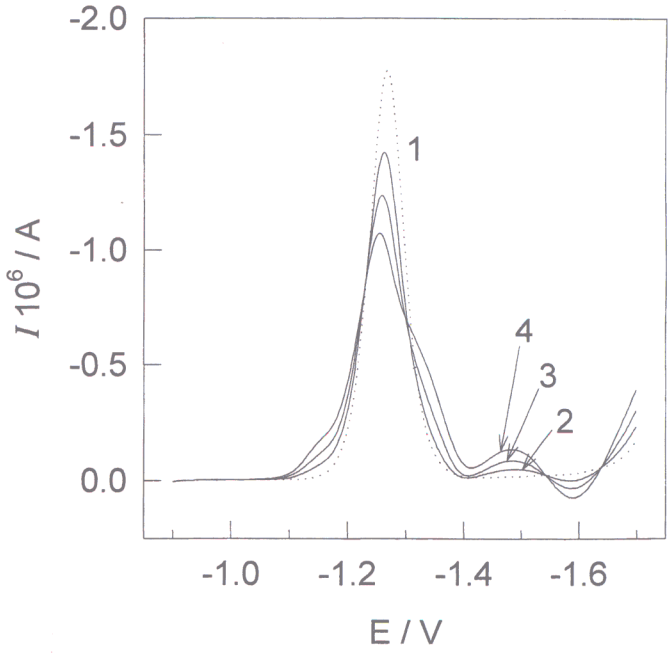

Fig. 6 *Modified Brdička method for the determination of MT content isolated from the digestive gland of cadmium-exposed mussels M. galloprovincialis.* Curve 1 represents the reduction of Co^{2+} to Co^{o} in the absence of MT. Curves 2, 3 and 4 were recorded after equal aliquots (10 µl each) of pooled MT-10(V) fraction, which contains MT and not DTT, were added. MT content is evaluated from the height of the signal at -1.5 V, while the signal related to DTT is not observed.

the MT content according to the modified Brdicka method, but interferes during the amperometric titration. The assumption was that dithiotreitol (DTT) acts as the ligand for Cd^{2+} ions during the titration experiments. On figs. 5 and 6 the characteristic current-potentials curves in a DPV mode are presented by which the MT content is determined according to the modified Brdička method. In both figs. 5 and 6 the signal assigned as curve 1 represents the reduction of Co^{2+} to Co^{0} and serves as the internal control that the measuring system is of acceptable purity. After the additions of the aliquots of the chromatographic fractions more than one signal is observed. The curves 2 to 4 in fig. 5 refer to the addition of a single chromatographic fraction MT-10(V-32) which contains MT and DTT. In fig. 6 the curves 2 to 4 refer to the addition of the pooled chromatographic fraction MT-10(V) which contains MT only, while DTT was beforehand removed. For the evaluation of the MT content the height of the catalytic hydrogen wave at -1.50 V is evaluated and related to the overall MT concentration. When DTT is present (Fig. 5) it is noticeable that at -1.17 V the signal exists. It disappears (Fig. 6) when DTT is removed from the chromatographic fraction. In such a manner we were able to detect the presence of DTT in the studied chromatographic fraction and to identify the interfering ligand.

Well-defined DTT concentration (2.5×10^{-6} M) was titrated with $CdCl_2$. The titration was performed in the concentration range from 2.5×10^{-8} M to 1.25×10^{-7} M $CdCl_2$ and the selected voltammograms are

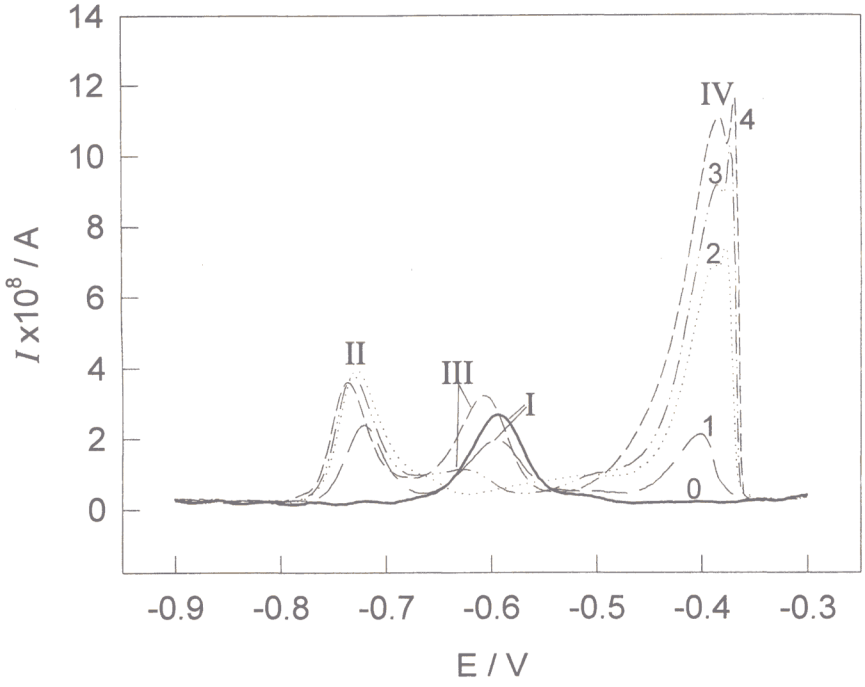

Fig. 7 *Current-potential curves in 0.59 M NaCl, borate buffer pH 7.9 of 2.5 x 10^{-6} M DTT (curve 0).* The titration with $CdCl_2$ was performed in the concentration range from 2.5 x 10^{-8} M to 1.45 x 10^{-7} M. Presented are the voltammograms at c($CdCl_2$), curve 1) 2.5 x 10^{-8} M; 2) 7.5 x 10^{-8} M; 3) 9.5 x 10^{-8} M; 4) 1.25 x 10^{-7} M.

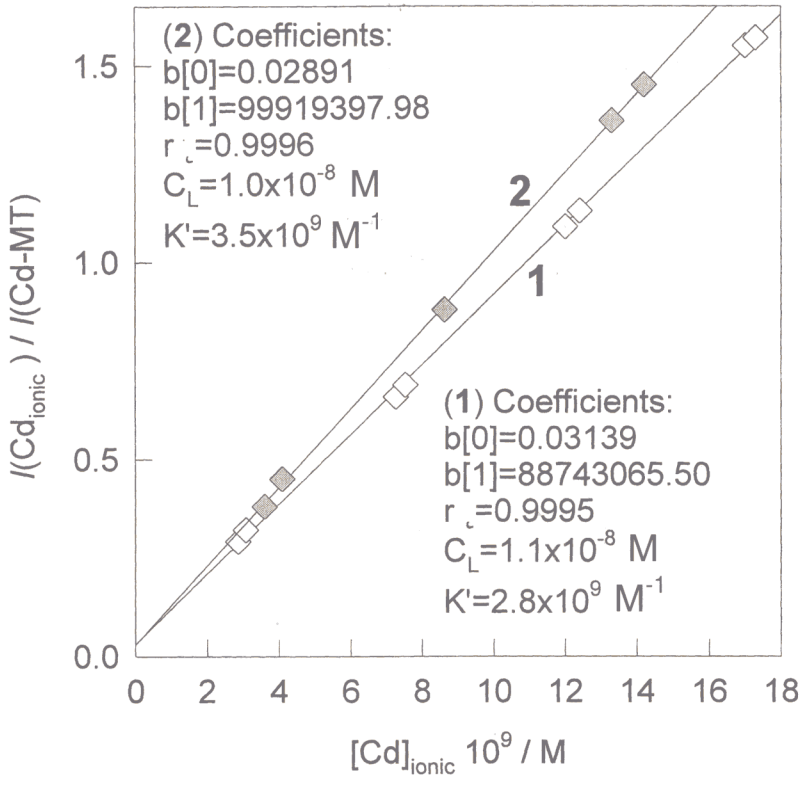

Fig. 8 *Plot of the ratio of the ionic cadmium peak current (ICd_{ionic}) to the peak current of cadmium complexed by MT ($ICd_{complexed}$) versus the ionic cadmium concentration.* The electrolyte composition and the measuring conditions are the same as reported in figs. 2 and 3. The straight-lines 1) and 2) represent two independent titrations with the defined c($CdCl_2$) of 50 µl of MT-10(V) pooled chromatographic fraction, which contains MT while DTT was removed.

presented in fig. 7. It is obvious that the signal I, recorded at -0.58 V vs. the reference electrode corresponds to the uncomplexed, electroactive ligand DTT. Before the addition of $CdCl_2$ only one signal exists (Fig. 7, curve 0). As the titration commences, the peak height of signal I decreases while the signal II appears and increases; signal II corresponds to the Cd-DTT complex. When signal II attains the maximum the signal I disappears and the signal III corresponding to the labile, ionic cadmium forms is observed. While titrating the defined concentration of DTT the same interrelation between different groups of signals exists as presented in figs. 2 and 3, which refer to the titration of chromatographic fractions MT-20(14) and MT-20(18) containing beside MT DTT, too. In the mentioned chromatographic fractions DTT is present at orders of magnitude higher concentration than MT and acts as the ligand for the added Cd^{2+} ions. It means that under such conditions it is not possible to study the cadmium-binding by MT. The interfering ligand i.e. DTT which is frequently used as the reducing agent in isolation and purification of MTs, has to be removed from the fraction intended for studying cadmium-binding to MT. The disadvantage of the removal procedure is that it causes further dilution of the MT available for studying the interaction with cadmium. In the MT isolation and purification steps the advice is to use mercaptoethanol as the reducing agent instead of DTT.

DISCUSSION

After performing metal, metallothionein and complexing measurements with MT-20(14) and MT-20(18) fractions there was not enough sample to remove DTT and perform additional complexing measurements. DTT is removed eluting certain volume of the chromatographic fraction with 3.5 ml 0.020 M Tris-HCl buffer pH 8.6 on an DEAE-Sephadex A-25 column. Therefore, the results on cadmium-binding to MT of mussel origin in the pooled chromatographic fraction MT-10(V), from which DTT was removed, are discussed (Erk and Raspor, 1999). On the voltammograms (Erk and Raspor, 1999, Fig. 4A) three groups of signals are observed, i.e. II, III and IV. In the absence of DTT the signal I was not recorded. The plot of the data recorded by two independent titrations of MT of mussel origin with cadmium is presented in fig. 8. The linear relationship indicates that complexing of cadmium occurs with one type of functional groups on MT molecule and the application of eq. 1 is justified. The calculated C_L and K' values are presented in table 2 [MT-10(V)]. For the determined complexing parameters, by means of the computer program (Pižeta and Branica, 1997) the confidence limits for 95% level of confidence were calculated, too (Table 2). Based on two independent measurements, K' of Cd-MT complex of mussel origin was determined as 2.8×10^9 M^{-1} and 3.5×10^9 M^{-1}, respectively. Comparing K' reported in table 2 we could notice that the values for Cd-DTT complex [Table 2, MT-20(14) and MT-20(18)] are close to the values for Cd-MT complex [Table 2, MT-10(V)]. Different is the potential range in which two cadmium complexes are recorded; Cd-DTT complex is recorded in the potential range -0.68 to -0.71 V, while Cd-MT complex is recorded at -0.80 V (Erk and Raspor, 1999). On the other hand, a previous study on cadmium-binding to the commercially available MT-(I+II) from rabbit liver (Erk and Raspor, 1998) indicates that Cd-MT complex was recorded at -0.67 V at pH 7.9. Ruiz *et al.* (1995) published the pH titration data performed with cadmium complexed by the commercial MT-(I+II), MT-I and MT-II from rabbit liver. The linear dependence of the peak potential (E_p) of the complex was recorded on pH in the range from 11 to 4, so that E_p changes from -1.1 V to -0.6 V, respectively. At pH≈4 the complete dissociation of the cadmium complex occurs.

Based on the results by Diaz-Cruz *et al.* (1997) on cadmium-binding properties of glutathione (GSH) it can be noticed that E_p of the Cd-GSH complex is at -0.65 V (at pH 7.0) and -0.71 V (at pH 8.5). Further on the data reported by Wagner-Roos *et al.* (1989) indicate that the peak of the cadmium-mercapto complexes (with L-cysteine, GSH and the synthetic model peptide containing SH-group) is at about -0.7 V.

The E_p at which different cadmium-mercapto complexes are recorded is predominantly at about -0.7 V. Such conclusion imposes a significant caution in the interpretation of the electrochemical data and points out to the necessity to check that the chromatographic fraction does not contain the interfering substance like DTT which masks the Cd-MT interaction.

From table 2 it could be noticed that the available ligand concentration (C_L) for complexing Cd^{2+} ions is reliably determinted while the confidence limits are narrow (2 to 7%). Further on, in agreement with the observation by Pižeta and Branica (1997) it follows that the apparent concentration stability constants (K') are not reliably determined due to broad confidence limits (100 to 300%). According to the recommendations by Ružić (1982) the stability constant of the complexed cadmium could be more accurately determined if K' is evaluated from the lower range of the titration curve, with small amounts of the titrant added. Considering the evaluation procedure for the complexing parameters it is clear that C_L is determined with higher accuracy than K', because K' is determined from the intercept (Eq. 1), taking into account C_L, too, which leads to the propagation of the error. Besides, the smaller the intercept, the higher the stability constant. If the intercept on the ordinate is very small, as usual for complexes of high stability constants, as is the case in this study, too, the measurements are made over a low range of the titrant.

As Pižeta and Branica (1997) discussed, the product of C_L and K' gives an indication whether the conditions are optimal for the evaluation of the complexing parameters. The lower limit is defined when the product is equal or lower than 1. Under such conditions it is difficult to state that the complexing occurs, even if the titration points and the measuring conditions are optimaly selected. The upper limit is defined when the product is equal or higher than 1000 (see also Ružić, 1982). From our experience the optimal range for the reliable amperometric titrations of the chromatographic fractions with Cd^{2+} ions is when the product C_L and K' is in the range 10 to 100, considering C_L in the range from 10^{-8} M to 10^{-7} M and K' at about 10^9 M^{-1}. Small amounts of Cd^{2+} ions must be added in order to collect a number of data sets for the evaluation of K'. The complexing parameters C_L and K' are evaluated from the ratio ICd_{ionic} / $ICd_{complexed}$. Therefore, it is important to dispose with sufficient number of data sets in the range where the ligand is close to the saturation, with small amounts of Cd_{added}, in order to evaluate ICd_{ionic}, too. In table 2 the product of C_L and K', determined for Cd-DTT in the chromatographic fractions MT-20(14), MT-20(18) and for Cd-MT determined in the pooled chromatographic fraction MT-10(V), is presented. The product amounts 30 to 80, which means that the titration and evaluation is performed in the optimal concentration range of the ligand and the apparent stability constant.

In the previous section the determination of K' from the intercept of the straight-line (Eq. 1) and its reliability, was discussed. In addition, an independent approach has been applied (Raspor and Pavičić, 1997) in order to calculate K' of Cd-MT complex from two independent measurements of linearized data presented in fig. 8. The apparent concentration stability constant of Cd-MT is defined as follows:

$$K' = [Cd\text{-}MT] / \{[Cd_{ionic}] (C_L - [Cd\text{-}MT]\} \quad (2)$$

Due to the fact that the amperometric titration is performed in a buffered solution of constant pH, the ligand deprotonation / protonation reactions are constant. As indicated in eq. 2, the concentration of MT not complexed with cadmium is calculated as the difference between the complexing capacity C_L, which defines operationaly available MT concentration for binding Cd^{2+} ions, and the concentration of cadmium bound to MT. Applying eq. 2 from each experimental point (Fig. 8) K' was calculated. The mean K' and the confidence limits for 95% level of confidence are for:

- measurement 1): 8 data sets $(3.19 \pm 1.11) \times 10^9$ M^{-1} and
- measurement 2): 5 data sets $(3.74 \pm 1.50) \times 10^9$ M^{-1}.

Comparing the values of K' calculated according to eq. 1 (Table 2) with those calculated according to eq. 2 (see above) satisfactory agreement is established. By the latter procedure K' is determined more reliably, as indicated by the narrower confidence limits (40%). The independent calculation of the stability constants according to eq. 2 clearly indicates that K' is the apparent concentration stability constant, because in the denominator neither the metal nor the ligand are expressed as the free chemical forms. By the additional statistical treatment of the data (Taylor, 1987) it has been proved that the two mean K' do not disagree at 0.05 significance level. Therefore, the pooled mean and the standard deviation were calculated and amount to $(3.4 \pm 1.3) \times 10^9\ M^{-1}$.

For MT of mussel origin and its interaction with cadmium we have no possibility to compare the reported data with those determined by some other technique(s) because to our knowledge such data do not exist in the literature, yet. At this stage we can only compare the reported data with another set of data on the apparent stability constant for Cd-MT complex, where MT refers to the commercially available MT-(I+II) (Erk and Raspor, 1998). Both studies were performed under the same physico-chemical and electrochemical conditions; $K'_{Cd.MT}$ [for MT-(I+II) rabbit liver] amounts to $7.6 \times 10^8\ M^{-1}$, which indicates that K' for the interaction of cadmium and metallothionein of mussel origin is of higher value.

CONCLUSIONS

The purified chromatographic fractions isolated norm the digestive gland of cadmium-exposed mussels (*Mytilus galloprovincialis*) were titrated with Cd^{2+} ions and recorded in a DPASV mode, in order to determine the complexing parameters C_L and K' of Cd-MT complex. The applied voltammetric method has the advantage that the formation of Cd-MT complex is measured in the homogeneous aqueous phase at the trace level of metal and ligand. The advantage of performing the complexing studies at the trace level of MT is to avoid insolubility and adsorption phenomena.

Caution is needed when Cd-MT interaction is measured in the purified chromatographic fraction which contains DTT as the reducing agent. During the titation DTT competes as the ligand for Cd^{2+} ions and masks Cd-MT complex formation. In order to study Cd-MT formation DTT should be removed from the chromatographic fraction. Preferably, mercaptoethanol should be used instead.

Evaluation of the amperometric titration data of pooled chromatographic fraction MT-10(V), from which DTT has been removed, indicates that C_L, i.e. the operationaly defined available MT concentration for complexing Cd^{2+} ions is reliably determined with the confidence limits 2 to 7%. Under the defined experimental conditions C_L amounts to 1×10^{-8} M. In 0.59 M NaCl, pH 7.9, 25°C the pooled mean and the standard deviation of the apparent concentration stability constant K' for Cd-MT complex, where MT refers to mussel digestive gland, amounts to $(3.4 \pm 1.3) \times 10^9\ M^{-1}$.

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