Possibilities of electrochemical techniques in metallothionein and lead detection in fish tissues

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ABSTRACT: In the present paper, we report on the use of adsorptive transfer stripping technique in connection with chronopotentiometric stripping analysis for metallothionein determination and of differential pulse anodic stripping voltammetry for lead detection in tissues of wild perch (*Perca fluviatilis*, *n* = 6) from the Svratka River in Brno, Czech Republic. Primarily, we determined the content of MT in tissues (muscles, gonads, liver and spleen) of perch. We measured the highest content of MT in spleen and liver (100–350 ng MT per gram of fresh weight). We assume that the content of MT determined in perch tissues is probably related with the age of the fish and, therefore, with their exposition to heavy metals naturally occurring in the Svratka River. We detected a lead concentration in the tissues of one perch. It clearly follows from the results that the content of MT well correlates with the concentration of lead.

Keywords: electrochemical detection; catalytic signal; peak H; heavy metals; fish; environmental pollution

Metallothionein (MT), which was discovered in 1957 by Margoshes and Valee as a part of an extract from the horse kidney (Margoshes and Vallee, 1957), belongs to a group of intracellular proteins. Four groups of metallothioneins (MT1, MT2, MT3 and MT4) have been known until now according to the Expert Protein Analysis System (ExPASy) Proteomics Server. Their molecular weight varies

Supported by Czech Science Foundation (Grant No. 526/07/0808), Grant Agency of the Academy of Sciences of the Czech Republic (Grant No. GAAV IAA401990701), and Ministry of Education, Youth and Sports of the Czech Republic (No. MSMT 6215712402).

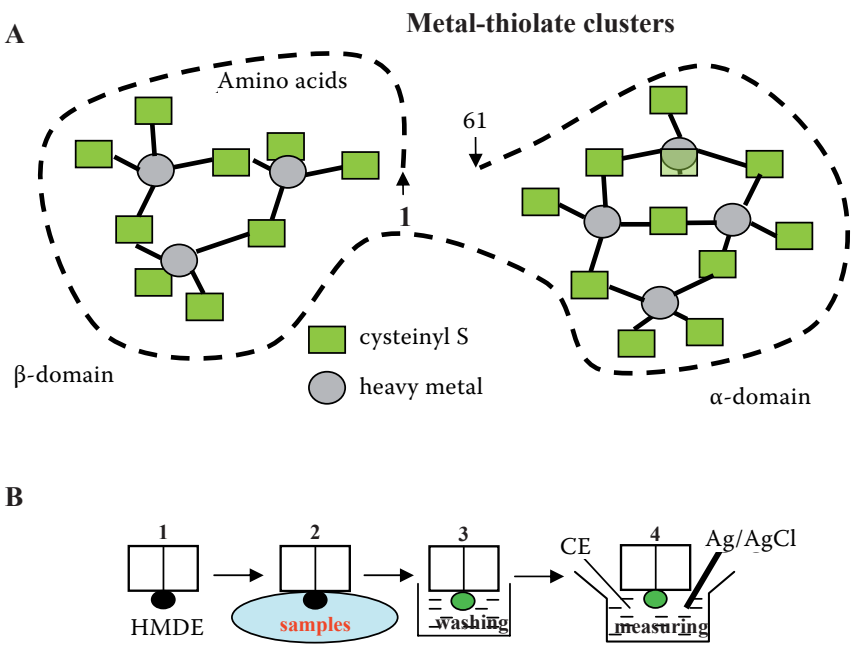

Figure 1. Scheme of techniques used for metallothionein analysis (**A**). Model of Metal-thiolate clusters (**B**)

from 6.0 to 6.9 kDa, the number of amino acids is about 61 and their pI is about 8.3. Only MT3 group (Palmiter et al., 1992; Kameo et al., 2005) differs from the others because it contains 68 amino acids and its pI is about 4.8. Although the members of the MT family were discovered nearly 40 years ago, their functional significance remains obscure. MTs are involved in many pathophysiological processes such as metal ion homeostasis and its detoxification, scavenging of reactive oxygen species, cell proliferation and apoptosis, chemo-resistance and radiotherapy resistance (Doki and Monden, 2004; Nordberg, 2004; Theocharis et al., 2004; Průša et al., 2005). Considering the heavy metal detoxification significance of MTs, these proteins can serve as biomarkers of heavy metal pollution of the environment (Raspor et al., 2004; Swierzcek et al., 2004; Ivankovic et al., 2005; Zorita et al., 2005). On the other hand, a comparison between the content of heavy metals and determined MTs could be very useful not only from toxicological aspects but also from biochemical aspects due to the better understanding of different functions of MTs in an organism (Figure 1A).

These data demonstrate the necessity to use analytical techniques not only for determination of MTs but also for detection of heavy metals in organisms of interest. Based on the recently published papers applying an electrochemical technique for determination of both heavy metals and metallothioneins (Kizek et al., 2001; Dabrio et al., 2002; Erk et al., 2002; Trnková et al., 2002; Ivankovic et al., 2003; Strouhal et al., 2003; Průša et al., 2004; Šestáková and Navrátil, 2005; Petrlová et al., 2006), we decided to use them for the same purposes here. Here, we report on the use of adsorptive transfer stripping technique in connection with chronopotentiometric stripping analysis (AdTS CPSA) for the determination of metallothionein and of differential pulse anodic stripping voltammetry for the detection of lead in tissues of wild perch (*Perca fluviatilis*) from the Svratka River in Brno, Czech Republic, because the knowledge of metallothionein content in an organism exposed to lead is scarce (Scortegagna et al., 1998; Atli and Canli, 2003; Gillis et al., 2004).

MATERIAL AND METHODS

Chemicals

Rabbit liver MT (MW 7 143), containing 5.9% Cd and 0.5% Zn, was purchased from Sigma Aldrich (St. Louis, USA). Tris(2-carboxyethyl)phosphine (TCEP) was produced by Molecular Probes (Evgen, Oregon, USA). The other chemicals were purchased from Sigma Aldrich. The stock standard solutions of MT at 10 µg/ml with 1mM TCEP were prepared with ACS water (Sigma-Aldrich, USA)

and stored in the dark at –20°C. Working standard solutions were prepared daily by the dilution of stock solutions. The pH value was measured with a WTW inoLab pH meter (Weilheim, Germany). The pH-electrode (SenTix-H, pH 0–14/3M KCl) was regularly calibrated by a set of WTW buffers (Weilheim, Germany).

Sample preparation for metallothionein analysis

Six perches (about 10–20 cm in length) were fished out in the Svratka River. Samples for the determination of MT (*Perca fluviatilis*; approximately 0.2 g of a tissue) were prepared according to the procedure reported by Kizek (Kizek et al., 2001). Briefly, the sample was kept at 99°C in a compact thermomixer (Eppendorf 5430, USA) for 15 min with occasional stirring, and then cooled to 4°C. The denatured homogenates were centrifuged at 15 000 g for 30 min at 4°C (Eppendorf 5402, USA). Then, the supernatant was analyzed.

Sample preparation for lead analysis

Recently we proposed an electrochemical method for the detection of lead in biological material (Vacek et al., 2004). Briefly, samples (*Perca fluviatilis*; approximately 0.2 g of a tissue) were transferred to a test tube and frozen by immersion in liquid nitrogen to disrupt the tissues. Then the extract was added to 1 ml of 0.2M phosphate buffer (pH 7.0), homogenized by vortexing for 15 min (Scientific Industries, Vortex–2 Genie, USA), and finally sonicated for 5 min at 200 W (Bandelin, Germany). The homogenate was then centrifuged at 14 000 g, 30 min at 4°C (Eppendorf 5402, USA). The supernatant (1 ml) was mixed with HCl (35%) for 30 min. After mixing, the deproteinized solution was neutralized with 13M NaOH prior to the analysis by differential pulse anodic stripping voltammetry.

Electrochemical measurements

Electrochemical measurements were performed with an AUTOLAB Analyser (EcoChemie, the Netherlands) connected to VA-Stand 663 (Metrohm, Switzerland), using a standard cell with three electrodes. The working electrode was a hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2. The reference electrode was an Ag/AgCl/3M KCl electrode and the auxiliary electrode was a graphite stick electrode. For smoothing and baseline correction the software GPES 4.4 supplied by EcoChemie was employed. An adsorptive transfer stripping technique (AdTS) (Húska et al., 2007) in connection with chronopotentiometric stripping analysis (CPSA) was used for the determination of metallothionein by recording the inverted time derivation of potential $(dE/dt)^{-1}$ as a function potential E (Figure 1B). Analysed metallothionein was reduced by 1mM TCEP according to Kizek et al. (2004) and Petrlová et al. (2006). CPSA parameters were as follows: I_{str} of –1 μA, temperature 20°C, supporting electrolyte 0.1M H_3BO_3 + 0.05M $Na_2B_4O_7$ (Sigma Aldrich, ACS).

Determination of lead by differential pulse anodic stripping voltammetry

The solution contained 1 ml of supporting electrolyte (0.1 M phosphate buffer, pH 5.5) and the sample prepared as described above was deoxygenated for 2 min by purging with water-saturated argon (99.999%) prior to measurements. Lead was deposited on the HMDE at a potential –0.6 V with the time of accumulation 60 s. During deposition the processing solution was stirred (1 450 rpm). The anodic scan was initialized at –0.6 V and stopped at 0 V. The step potential was 5 mV, modulation amplitude 50 mV, and time interval 0.24 s. Other experimental details are described in Vacek et al. (2004).

RESULTS AND DISCUSSION

Analysis of metallothionein by AdTS CPSA

MT was determined by AdTS CPSA at the stripping current of 1 μA because a higher stripping current decreased the response. MT (1 ng/ml) gave a well reproducible signal at the potential of –1.70 V (Figure 2A). Similar catalytic signals of MT were obtained by other authors (Kizek et al., 2001; Trnková et al., 2002; Strouhal et al., 2003; Průša et al., 2004, 2005). In addition, we tested the influence of different times of accumulation on the height of MT signal. The obtained depend-

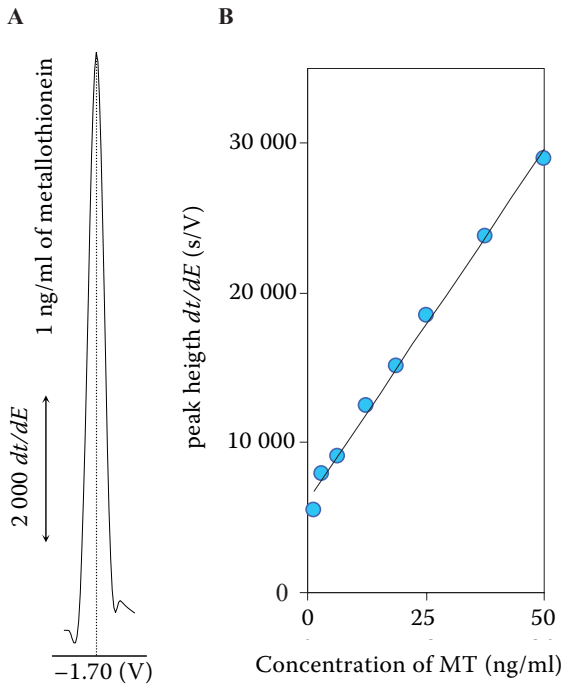

Figure 2. Determination of MT by adsorptive transfer technique in connection with chronopotentiometric stripping analysis. Chronopotentiogram of MT (1 ng/ml) (**A**). Dependence of the MT signal on its concentration (**B**) and on accumulation time (inset in B). The CPSA supporting electrolyte – 0.1M H_3BO_3 + 0.05M $Na_2B_4O_7$. AdTS CPSA parameters were as follows: initial potential of 0 V, end potential –1.85 V, temperature 20°C, accumulation time 90 s (except B). For other details see the Material and Methods section

ence is shown in Figure 2B. It follows from the result that the highest response was obtained at the accumulation time of 90 s. After that we optimized the optimal accumulation time, and we studied the dependence of MT peak height on the concentration of the target molecule. The calibration curve was not linear within the whole tested concentration interval as it is shown in the inset in Figure 2B. Therefore the curve was split into two parts: 0–1.2 ng MT/ml (y = 14 386x + 129.55; R^2 = 0.9916) and 2–20 ng MT/ml (y = 2 134.2x + 24 520; R^2 = 0.996), n = 3, R.S.D. = 8%, where we obtained sufficient linearity with R^2 of the calibration curves higher than 0.99.

Electrochemical determination of metallothionein and lead in tissues of perch (*Perca fluviatilis*)

We used the above-mentioned optimized technique for the determination of MT in tissues of perch (*Perca fluviatilis*). Six perches (about 10–20 cm in length) were fished out in the Svratka River. The perches were healthy and have not been treated by any control concentration of heavy metals. The tissues (muscles, gonads, liver and spleen) were frozen (–20°C), and then prepared prior to measurements as described in the Materials and Methods section. We determined the highest concentration of MT in spleen and liver (100–350 ng MT/g). It has been published that metals are intensively metabolised in gonads because the most effective mechanisms of heavy metal detoxification take place there and the content of MT would be higher in comparison with the other tissues (Nordberg and Nordberg, 2000; Nordberg et al., 2000). We found out that the concentration of MT in the perch gonads was about 25–250 ng MT/g. Moreover, due to a low detection limit of our detection method we were able to determine MT in the tissues (muscles) in which it has not been done yet. We observed that the concentration of MT was really low and varied from units to tens of ng of MT per gram of fresh weight. We assume that the concentration of MT determined in perch tissues is probably related with the age of the fish and, therefore, with their exposition to heavy metals naturally occurring in the Svratka River. To evaluate our assumption the determination of lead in the analysed tissues was needed. The experimental and field studies of the content of lead in fish and their hosts were done by Sures and others (Sures et al., 1997, 2000, 2003;

Table 1. Content of lead in tissues of perch (*Perca fluviatilis*; n = 5)

Tissue	Concentration of lead (ng per gram of fresh weight)*
Liver	273 ± 14
Gonads	625 ± 28
Spleen	362 ± 16
Muscles	84 ± 5

*Results are expressed as mean ± S.D.

Siddall and Sures, 1998; 1999, 2001; Zimmermann et al., 1999). We chose tissues from one analysed perch and detected lead there (Table 1). We found out that the highest content of lead was determined in gonads, followed by spleen, liver and muscles, where the concentration of lead was about 84 ± 5 ng per gram of fresh weight. It clearly follows from the results that the concentration of MT increased with the increasing concentration of lead (Table 1).

CONCLUSION

The monitoring of heavy metal ions in the environment represents one of the important markers of environment quality (Svobodová et al., 2002, 2004; Žlábek et al., 2005). The introduction of new markers of heavy metal pollution will enable us to better monitor threats of heavy metal intoxications. We showed that the MT concentration increased with the increasing content of lead in tissues of wild perch. It follows from the results that MT could be a marker of environmental pollution by lead.

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Received: 2007–02–24
Accepted after corrections: 2007–03–20

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