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ELECTROCHEMICAL DETERMINATION OF METALLOTHIONEIN IN THE DOMESTIC FOWL

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Metallothionein (MT) belongs to group of intracellular, low-molecular and cysteine-rich proteins with a molecular weight from 6 to 10 kDa. Owing to their high affinity to heavy metals (Zn, Cd, As, etc.) their main role is homeostatic control and detoxification of metal ions in an organism. In the present work we aimed at suggesting and utilizing electroanalytical techniques to determine content of MT in the blood serum of domestic fowls. Electrochemical measurements were performed with an AUTOLAB Analyser connected to VA-Stand 663, using a standard cell with three electrodes. Particularly, MT was detected by adsorptive transfer stripping technique in connection with differential pulse voltammetry. The detection limit of MT was estimated down to 100 fM (standards only) or down to 100 pM measured in the presence of blood serum. The average content of MT was 21.3 μM. The MT level in hens was about 25 % higher than in cocks. This phenomenon can be related to higher demands on the content of this protein in hens due the requirement for ion transport to form eggshell.

Key words: electrochemical method, differential pulse voltammetry, Brdicka reaction, protein, bird

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Metalotionein (MT) pripada grupi intracelularnih proteina male molekularne mase bogatih cisteinom, s molekularnom masom od 6 do 10 kDa. Zbog njihovog afiniteta prema teškim metalima (Zn, Cd, As, itd.) njihova glavna uloga je homeostatska kontrola i detoksifikacija iona metala u organizmu. U ovom radu predlažu se elektroanalitičke tehnike za određivanje sadržaja MT u krvnom serumu domaće peradi. Elektrokemijska mjerenja izvršena su uređajem AUTOLAB Analyser povezanim s VA-Stand 663, koristeći standardnu ćeliju s tri elektrode. Osim toga MT je određivan tehnikom adsorptivnog transfera, povezanoj s voltmetrijom diferencijalnog pulsa. Granica detekcije MT je procjenjivana do 100 fM (samo standardi) ili do 100 pM, mjereno u prisutnosti krvnog seruma. Prosječni sadržaj MT bio je 21.3 μM. Razina MT kod kokoši bila je otprilike 25% viša nego kod pijetlova. Ta pojava može se objasniti većom potrebom za ovim proteinom kod kokoši zbog transporta iona prilikom stvaranja ljuske jajeta.

Ključne riječi: elektrokemijska metoda, voltmetrija diferencijalnog pulsa, Brdicka-reakcija, protein, ptice

INTRODUCTION

Levels of metal ions are strictly maintained by specific peptides and proteins in organisms (KAGI *et al.*, 1988). Particularly, glutathione and metallothioneins are mainly responsible for regulation in animals, including bird species (HOPKINS, 1921; MEISTER *et al.*, 1983; KAGI *et al.*, 1988; SEN *et al.*, 1996; TOWNSEND *et al.*, 2003). Metallothioneins (MT) belong to a group of low molecular proteins rich in cysteine able to bind heavy metals to thiolated clusters (FOWLER, 1990; PALMITER, 1994; DABRIO *et al.*, 2002). The molecular mechanisms of the MT expression are less well understood. The currently accepted opinion is that synthesis can be activated by the presence of heavy metal ions within a cell (Fig. 1). Metallothioneins occur throughout the whole animal kingdom and are divided into several classes accordingly. MT I class comprises metallothioeneins occurring in mammalian species. All other protein non-mammalian metallothioneins are included in the MT II class. Metalloisopolypeptides with a gama glutamyl-cysteinyl moiety are called MT III class of metallothioneins.

Two groups of metallothioneins, MT1 and MT2, were determined in birds, however no exact data on the MT gene are available (ANDREWS *et al.*, 1996; VILLARREAL *et al.*, 2006). The most commonly used techniques for determination of MT in bird tissues are immunological. The levels of MT determined have tended to fluctuate (ELLIOTT *et al.*, 1992; SAVINOV *et al.*, 2003; TRUST *et al.*, 2000). These techniques were employed for the investigation of the relation between expression of MT gene and the level of heavy metal ions in kidney and liver tissues (NAM *et al.*, 2007), because the level of both toxic and essential heavy metals can correlate with state of a health of an animal (ELLIOTT *et al.*, 1992; ELLIOTT *et al.*, 1997; COSSON, 1989; TRUST *et al.*, 2000; BARJAKTAROVIC *et al.*, 2002; CHENG *et al.*, 2004; EK *et al.*, 2004a, EK *et al.*, 2004b, PARK *et al.*, 2004; NAM *et al.*, 2005a; NAM *et al.*, 2005b; KOJADINOVIC *et al.*, 2007). Based on the results obtained metallothionein can be considered a biomarker of heavy metal intoxication (ELLIOTT *et al.*, 1992; KUSHLAN, 1993; STLOUIS *et al.*, 1993; ELLIOTT *et al.*, 1997; SALDIVA *et al.*, 1998). In general, to detect level of MTs in vari-

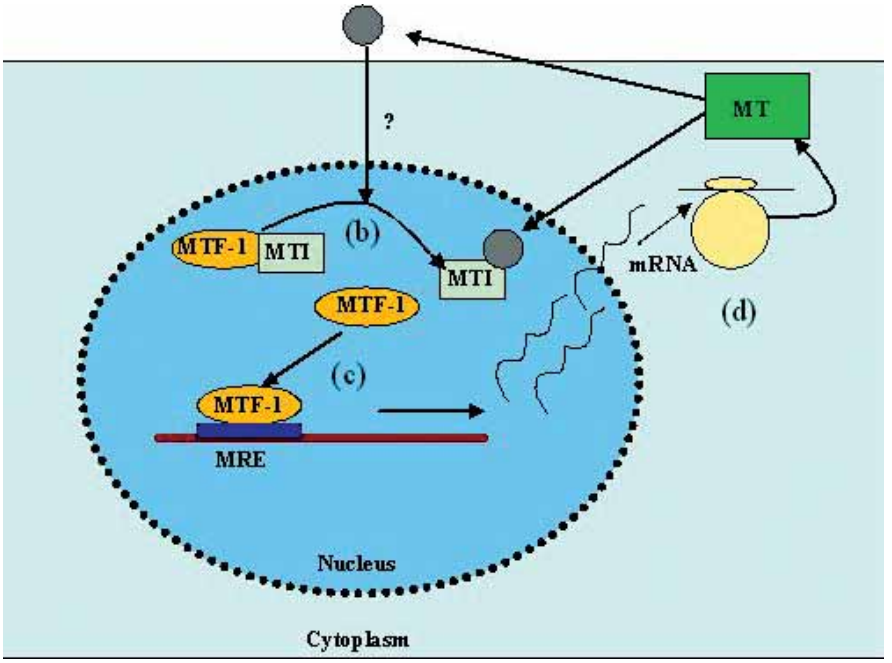

Fig. 1. **Cell regulation of heavy metal ions level.** A heavy metal ion enters through a cytoplasmic membrane of a cell via ionic channels or special transporters (**a**). After entering the cytoplasm, the ion interacts with a complex of metal-regulatory transcription factor-1 (MTF-1) and metal synthesis inhibitor (MTI) (**b**). The ion is bonded by MTI. Due to this MTF-1 is released and can bind to regulation sequence of DNA called metal responsive element (MRE) (**c**). Then, the gene responsible for synthesis of metallothioneins is transcribed. The synthesized mRNA molecule is translated into MT (**d**).

ous biological samples, techniques and methods divided to several groups can be used. The first group, such as Cd-hem, is based on quantification of metal ions bounded in MT molecules (MOFFATT *et al.*, 1997, YOSHIDA *et al.*, 1998). The second group of the methods quantifies total content of MTs in the target sample according to content of metal ions bonded in MTs. It is mainly spectrometric techniques that belong to this group (SZPUNAR *et al.*, 2003; SZPUNAR, 2005). The third group of the methods is based on detecting sulfhydryl moieties of MTs using their chemical labelling by Ellman reagent (VIARENGO *et al.*, 1997; JIANG *et al.*, 2000). The fourth group of methods uses antibodies against MTs. Among these methods, Western blotting, immunohistochemistry, immunofluorescence, radio-immunoanalysis (RIA) and enzyme-linked immunosorbent assay (ELISA) are included (CHU *et al.*, 2006; ALVARADO *et al.*, 2007). Separation techniques such as liquid chromatography or capillary electrophoresis belong to the fifth group of methods (BEATTIE, 1998; CHASSAIGNE *et al.*, 1998). The sixth group of methods is based on the detection of mRNA (CHOI *et al.*, 2007, ONO *et al.*, 2007).

However the authors of all the above-mentioned reports killed or harmed the animal to determine MT level because sampling of tissues was invovled. This approach is not suitable for long-time monitoring of the state of health of an animal of interest. An electrochemical determination of metallothionein is a seventh group of analytical methods and techniques used for detection of these proteins and has been discussed in several reports (KIZEK *et al.*, 2001; RASPOR *et al.*, 2001; STROUHAL *et al.*, 2003; RASPOR *et al.*, 2004; PETRLOVA *et al.*, 2006). However electrochemical techniques have not been employed yet to determine MT in body liquids of bird species. The aim of this paper was firstly to use an electrochemical method for the measurement of MT level in birds. Domestic fowl was used as model organism.

MATERIAL AND METHODS

Chemicals and instruments

Rabbit liver MT (MW 7143), containing 5.9 % Cd and 0.5 % Zn, was purchased from Sigma Aldrich (St. Louis, USA). Tris(2-carboxyethyl)phosphine (TCEP) is produced by Molecular Probes (Evgen, Oregon, USA). $Co(NH_3)_6Cl_3$ and other used chemicals were purchased from Sigma Aldrich in ACS purity unless noted otherwise. The stock standard solutions of MT at 10 μg/ml were prepared with ACS water (Sigma-Aldrich, USA), reduced by adding 1 mM TCEP (KIZEK *et al.*, 2004) and stored in the dark at –20 °C. Working standard solutions were prepared daily by dilution of the stock solutions. Deionised water underwent demineralization by reverse osmosis using the instruments Aqua Osmotic 02 (Aqua Osmotic, Tisnov, Czech Republic) and then it was subsequently purified using Millipore RG (Millipore Corp., USA, 18 MΩ) – MiliQ water.

Biological material

Blood serum samples of forty domestic fowls *Gallus gallus f. domestica* (20 females and 20 males, Jinacovice Game Bird Farm, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic) were used for analyses. Before blood collecting the weight and age of the animals were determined. Blood was collected during routine health status check from *vena ulnaris* or *vena jugularis* using a hypodermic needle and a syringe in a volume not threatening to the animal. Heparin (HEPARIN LÉČIVA 5000 iu/1 ml) was used as an anticoagulant. Immediately after collection, blood was centrifuged for 10 min at 3,000 rpm. Then, plasma was separated and frozen in 1.5 ml Eppendorf vials prior further analysis.

Preparation of biological samples for electrochemical analysis

The sample was prepared by heat treatment. Briefly, the sample was kept at 99 °C in a thermomixer (Eppendorf 5430, USA) for 15 min with occasional stirring, and then cooled to 4 °C. The denatured homogenates were centrifuged at 4 °C, 15,000 *g* for 30 min (Eppendorf 5402, USA). Heat treatment effectively denatures and removes high molecular weight proteins from samples (KIZEK *et al.*, 2001; ERK *et al.*, 2002; PETRLOVA *et al.*, 2006). Supernatants were stored at –20 °C.

Differential pulse voltammetry using Brdicka reaction

Electrochemical measurements were performed with a VA Stand instrument (Metrohm, Switzerland), connected to AUTOLAB (EcoChemie, Netherlands), using a standard cell with three electrodes. A hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2 was the working electrode. An Ag/AgCl/3M KCl electrode was the reference and glassy carbon electrode was auxiliary electrode. The supporting electrolyte (1 mM $[Co(NH_3)_6]Cl_3$ and 1 M ammonium buffer; NH_3(aq) and NH_4Cl, pH 9.6) was changed after five measurements. The DPV parameters were as follows: initial potential of –0.7 V, end potential of –1.75 V, modulation time 0.057 s, time interval 0.2 s, step potential 2 mV, modulation amplitude –250 mV, E_{ads} = 0 V. All experiments were carried out at a temperature of 4 °C (Julabo F12, Germany). Volume of diluted sample for analysis was 1 µl. For smoothing and baseline correction the software package GPES 4.9 supplied by EcoChemie was employed.

pH measurements

The pH value was measured using WTW inoLab Level 3 with terminal Level 3 (Weilheim, Germany), controlled by a personal computer program (MultiLab Pilot; Weilheim, Germany). The pH-electrode (SenTix-H, pH 0–14/3M KCl) was regularly calibrated by a set of WTW buffers (Weilheim, Germany).

Descriptive mathematics

MICROSOFT EXCEL® (USA) was used for statistical analyses. Results are expressed as mean ± S.D. unless noted otherwise.

RESULTS AND DISCUSSION

The blood samples of domestic fowls were processed according to the procedure mentioned in the Materials and Methods section. Measurements were carried out with electrochemical analyser by adsorptive transfer technique coupled with a differential pulse voltammetry Brdicka reaction. Briefly, a sample (volume of 5 µl) was accumulated on the surface of the working electrode for 120 s. Then, the electrode was rinsed. The electrode was transferred to pure supporting electrolyte containing cobalt (III) ions. A typical DP voltammogram is shown in Fig. 2. In the voltammogram several peaks can be observed. The peak at a potential of –1.0 V corresponds to the formation of a cobalt-protein complex. Another peak at –1.25 V represents first catalytic signal hydrogen evolution from the supporting electrolyte catalyzed by a presence of a protein. The last peak at –1.45 V is the second catalytic signal hydrogen evolution from the supporting electrolyte catalyzed by a presence of a protein. Based on our previously published results the signal called Cat2 is suitable for the quantification of the MT content (PETRLOVA *et al.*, 2006).

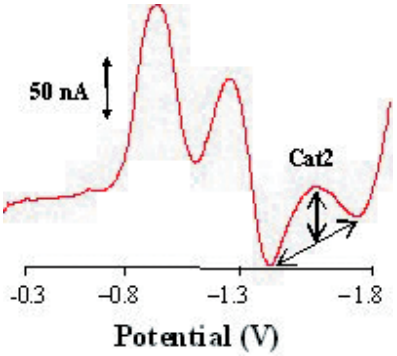

Fig. 2. Typical DP voltammogram of blood serum sample from domestic fowl. The signal called Cat2 was used to quantify MT.

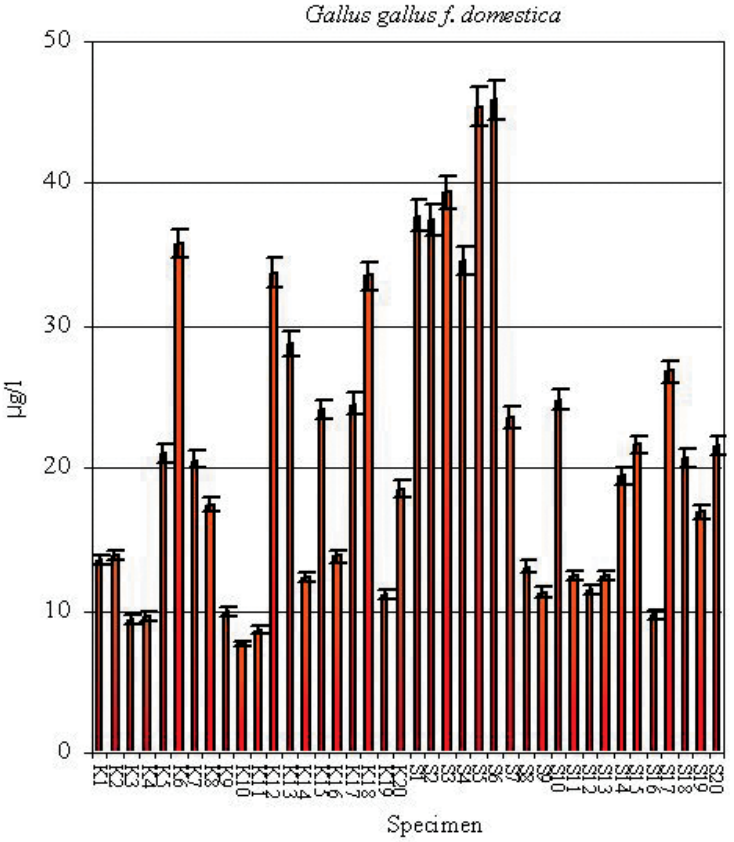

Fig. 3. Content of metallothionein in the blood serum samples, K-cocks, S-hens.

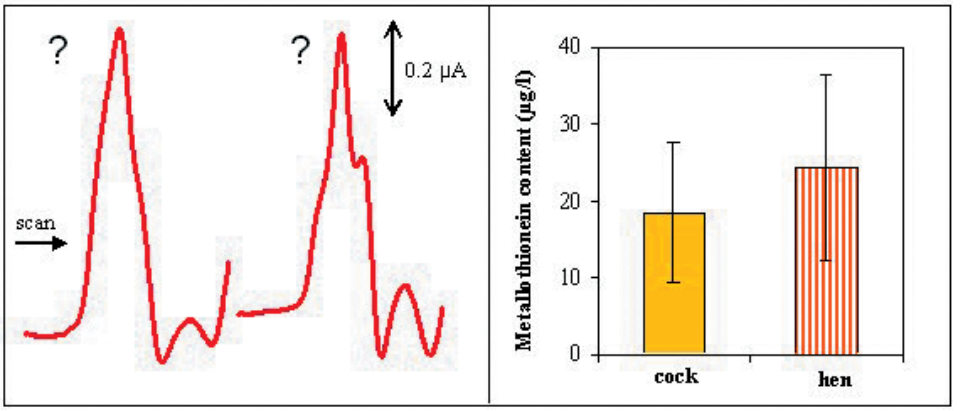

Fig. 4. Typical DP voltammograms of cock and hen blood serum sample (**A**). Average content of metallothionein in cocks (n = 20) and hens (n = 20) (**B**).

The level of MT was determined in forty domestic fowls (20 females and twenty males). The results obtained are shown in Fig. 3. It clearly follows from the results obtained that the MT contents in blood of the animals differ. The level of MT varied from 6 to 35 µg of MT per l. This phenomenon can be related to the state of health of the specimen and other factors not fully recognized. However. the relative standard deviation of a measurement was about 4 %. The average level of MT at the animals was 21.3 ìg of MT per l.

Typical DP voltammograms of hens and of cocks are shown in Fig. 4. It follows that the voltammetric curves differ from each other. If we mathematically treated the data (comparison of the signals measured at specimens), we could distinguish the voltammograms of a hen and a cock. Moreover the average levels of MT in a hen and a cock were 24 ± 12 µg MT/l and 18 ± 9 µg/l, respectively. The MT level in hens was about 25 % higher than that in cocks. In addition the average levels of MT significantly differed between hens and cocks at p = 0.1. This phenomenon can be related to higher demands on the content of this protein in hens because of the requirement to provide ion transport for the formation of eggshell.

CONCLUSION

The level of metallothionein probably plays an important role in the metabolism of gallinaceous birds due to their food habits. Moreover metallothioniens are probably involved in the transport of important ions during the formation of eggshell.

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