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EMPLOYING VOLTAMMETRY FOR DETERMINATION OF LOW MOLECULAR MASS THIOLS AND METALLOTHIONEIN IN BLOOD OF PIG (*SUS SCROFA DOMESTICA*)

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Beklova, M., Adam, V., Mares, P., Zeman, L., Dolezal, P., Pikula, J., Trnkova, L. & Kizek, R.: Employing voltammetry for determination of low molecular mass thiols and metallothionein in blood of pig (*Sus scrofa domestica*). Nat. Croat., Vol. 17, No. 4, 293–301, 2008, Zagreb.

Metallothioneins (MT) play a key role in maintaining the homeostasis of essential metals and in protecting of cells against metal toxicity as well as cell oxidative damaging. The aim of this work is to propose a new approach for processing a biological sample for analysis of thiols including metallothioneins. Moreover, the proposed procedure is tested on quantification of MT and total thiol content in blood serum of pig (*Sus scrofa domestica*), which has not been previously been performed. The blood serum (10 μl) was collected and transferred to 0.2 M phosphate buffer (990 μl). The sample (100 × diluted) was placed in a thermomixer, where heat denaturation of most of the proteins proceeded. The processed blood serum sample was electrochemically measured to determine total

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content of thiols (cysteine, glutathione, metallothionein and other low molecular thermostable thiols) and content of MT. The average level of the thiols and MT were estimated as 165 ± 20 μM and 5.2 ± 0.6 μM, respectively.

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

Beklova, M., Adam, V., Mares, P., Zeman, L., Dolezal, P., Pikula, J., Trnkova, L. & Kizek, R.: Uporaba voltmetrije za određivanje tiola i metalotioneina male molekularne mase u krvi svinje (*Sus scrofa domestica*). Nat. Croat., Vol. 17, No. 4, 293–301, 2008, Zagreb.

Metalotioneini (MT) igraju ključnu ulogu u održavanju homeostaze esencijalnih metala i u zaštiti od toksičnih metala te od oštećivanja stanice oksidacijom. Cilj ovog rada je predložiti novi pristup obradi bioloških uzoraka za analizu tiola, uključujući metalotioneine. Osim toga, predloženi postupak se testira pri kvantifikaciji MT i ukupnog sadržaja tiola u krvnom serumu svinje (*Sus scrofa domestica*), što se dosad nije radilo. Prikupljan je krvni serum (10 μl) te prebacivan u 0.2 M fosfatni pufer (990 μl). Uzorak (100 × razrijeđen) je stavljen u termomikser gdje se nastavila toplinska denaturacija većine proteina. Obrađeni uzorak seruma je elektrokemijski izmjeren da bi se odredio ukupni sadržaj tiola (cistein, glutation, metalotionein i drugi termostabilni tioli male molekularne mase) te sadržaj MT. Prosječna razina tiola i MT su procijenjeni na 165 ± 20 μM, odnosno 5.2 ± 0.6 μM.

Ključne riječi: elektrokemijska metoda, voltmetrija diferencijalnim pulsom, Brdicka-reakcija, protein, svinja

INTRODUCTION

Thiols can be defined as a group of compounds rich in –SH moieties. The thiol moiety is highly reactive and is often found conjugated to other both organic and inorganic molecules. One of the main features of this moiety is its ability to bind metal ions tightly (MEISTER *et al.*, 1983; ANDERSON, 1985; MEISTER, 1988). Even so simple a molecule as glutathione (γ-glutamyl-L-cysteinylglycine, GSH) is able to interact with heavy metal ions and, thus, to regulate their level. In addition thiols can scavenge highly reactive radicals (MEISTER *et al.*, 1983; ANDERSON, 1985; MEISTER, 1988). Metallothioneins (MT) represents other biologically important groups of thiols (KAGI *et al.*, 1988). These proteins are abundant through the whole animal kingdom, and they have also been found in higher plants, eukaryotic microorganisms and some prokaryotes (PALMITER, 1994). MT can be found mostly in parenchymatous tissues such as liver, kidney, pancreas and intestines in animal species. Moreover the MT level strongly depends on animal species, analysed tissue, age of the animal, eating habits and probably on other factors not yet fully understood and identified

The molecular characteristic of metallothioneins in pigs was firstly reported in 1998 E (HUANG *et al.*, 1998). MT have been investigated mainly due to their connection with the intake of both essential and toxic metals (WEBB *et al.*, 1975; VERMA *et al.*, 1978; STILLMAN *et al.*, 1986; YOSHIDA *et al.*, 1987; HENRY *et al.*, 1994; YOSHIDA *et al.*, 1997; MARTINEZ *et al.*, 2005; CARLSON *et al.*, 2007; FEKETE *et al.*, 2007). Metallothionein level can considerably affect the uptake of biologically important essential elements such as zinc (MARTINEZ *et al.*, 2004). Moreover metallothionein as a protein able to regulate the level of reactive oxygen species has been tested as an anti-stress drug. It was found that after its administration a decrease in influence of

stressors and enhancement of meat quality occur (LI *et al.*, 2006; LI *et al.*, 2007). Its pharmacological effects were also investigated in the re-oxidation of muscles (LI *et al.*, 1998).

To determine low molecular mass thiols in a biological sample precise analytical instruments with low detection limits are needed. Recently a number of analytical approaches and methods for the determination of biologically active thiol compounds have been developed (HANSEN *et al.*, 2007), including both stationary (e.g. voltammetry, chronopotentiometry, polarography) (ADAM *et al.*, 2007) and flow techniques (POSSARI *et al.*, 2006) (such as liquid chromatography (LC) (KAWAKAMI *et al.*, 2006), gas chromatography (GC) and capillary electrophoresis (CE)) (LAVIGNE *et al.*, 2007; YAO *et al.*, 2007). For detection of MT spectrometric (SZPUNAR, 2005), immunological (ALVARADO *et al.*, 2007), hyphenated (BEATTIE, 1998) and electrochemical (KIZEK *et al.*, 2001; STROUHAL et al., 2003) techniques are being used.

The aim of this work is to propose new approach to processing a biological sample prior to quantification of low molecular mass thiols and metallothioneins. Moreover the proposed procedure has been tested on quantification of MT and total thiol content in the blood serum of the pig (*Sus scrofa domestica*), which has not been previously performed.

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 chemicals used 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 of 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 seven female pigs (Farm of Mendel University of Agriculture and Forestry, Zabcice, Czech Republic) were used for analysis. Before blood collecting, the weight and age of the animals were determined. Blood was collected during routine health status check from *vena ulnaris* (from 1 to 5 ml) using a hypodermic needle and a syringe in a volume not threatening the animal. The blood was clotted. Subsequently, the clotted blood was centrifuged at 3,000 rpm for 10 min. (Eppendorf, Germany), the blood serum was obtained and immediately measured.

Adsorptive transfer stripping technique coupled with differential pulse voltammetry Brdicka reaction – MT content

Electrochemical measurements were performed with an AUTOLAB Analyser (EcoChemie, Netherlands) connected to VA-Stand 663 (Metrohm, Switzerland), using a standard cell and three electrodes. The working electrode was a hanging mercury drop electrode (HMDE). The reference electrode was an Ag/AgCl/3M KCl electrode and a glassy carbon electrode was used as the auxiliary electrode. Smoothing and baseline correction was employed by GPES 4.9 software supplied by EcoChemie. The principle of the adsorptive transfer stripping technique is based on the strong adsorption of the target molecule on the surface of the working electrode in an open circuit. The hanging mercury drop electrode is periodically renewed. Target molecules are adsorbed on the surface of the renewed working electrode in an open circuit. The electrode is rinsed with a supporting electrolyte. The electrode with the adsorbed target molecules is measured in the presence of the supporting electrolyte. MT was measured by AdTS coupled with differential pulse voltammetry (DPV) Brdicka reaction. Brdicka supporting electrolyte (1 mM $Co(NH_3)_6Cl_3$ and 1 M ammonia buffer (NH_3(aq) + NH_4Cl, pH = 9.6) were used without surface-active agent additives. AdTS DPV Brdicka reaction parameters were as follows: an initial potential of –0.35 V, an end potential of –1.8 V, a modulation time of 0.057 s, a time interval of 0.2 s, a step potential of 1.05 mV, a modulation amplitude of 250 mV, E_{ads} = 0 V. Temperature of supporting electrolyte was 4 °C.

Differential pulse voltammetry Brdicka reaction – Total thiol content

Electrochemical measurements were performed with a 747 VA Stand instrument connected to 746 VA Trace Analyzer and 695 Autosampler (Metrohm, Switzerland), using a standard cell with three electrodes and a cooled sample holder (4 °C). 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 a glassy carbon electrode was the 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) were 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 5 or 10 µl. For smoothing and baseline correction the software 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).

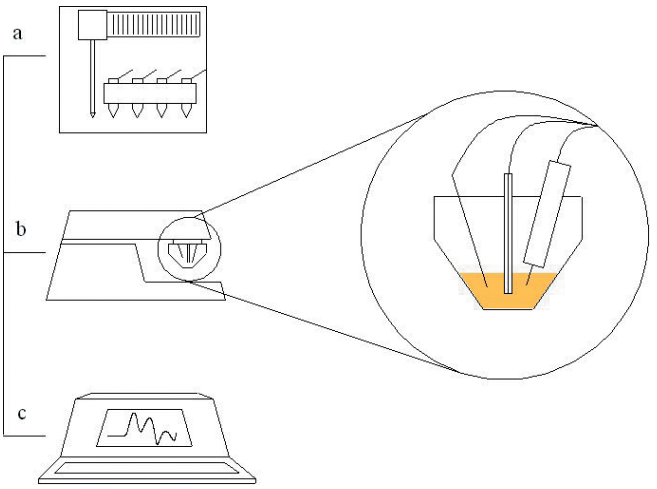

Fig. 1. Experimental arrangement of automated electrochemical analyser. a) sample holder, b) electrochemical cell using three electrodes, c) computer-controlled unit.

RESULTS AND DISCUSSION

It has been shown that thiols play crucial roles in numerous biologically important processes including regulation of metal ion level and scavenging of reactive oxygen species. Recently we published a paper in which we determined the metallothionein level of selected animal species bred in captivity (ADAM *et al.*, 2007). Association of changes in metallothionein level in blood of the animals and their taxonomic group was observed. Pigs belong among the omnivores. The level of thiols in this animal has not been investigated in great details (WEBB *et al.*, 1975; VERMA *et al.*, 1978; STILLMAN *et al.*, 1986; YOSHIDA *et al.*, 1987; HENRY *et al.*, 1994; YOSHIDA *et al.*, 1997; MARTINEZ *et al.*, 2005; CARLSON *et al.*, 2007; FEKETE *et al.*, 2007). In the present paper we aimed at determination of total thiol and MT content.

The procedure for the preparation of pig blood serum samples differed from the previously published protocols (PETRLOVA *et al.*, 2006). The blood serum (10 μl) was collected and transferred to 0.2 M phosphate buffer (990 μl). The sample (100 × diluted) was placed in a thermomixer, where heat denaturation of most of the proteins proceeded. Denatured proteins were removed from the solution by centrifugation at 16 000 g for 30 min. The supernatant obtained was used for electrochemical measurements. The advantage of the newly proposed sample processing is the much lower consumption of a sample (microlitres). The other advantage of the processing is the better denaturation of thermolabile proteins in excess of the buffer. If we denatured a sample without dilution by the buffer, content of denaturing proteins was higher and obtaining of supernatant was more difficult.

The processed blood serum sample was electrochemically measured to determine total content of thiols (cysteine, glutathione, metallothionein and other low

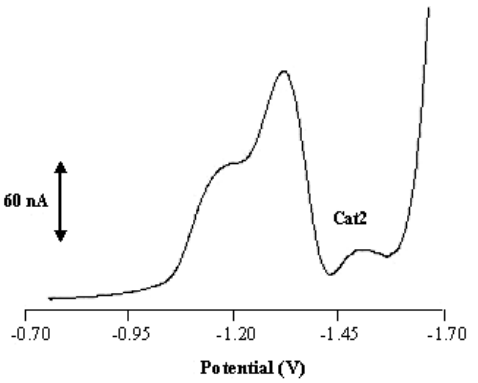

Fig. 2. Typical DP voltammogram of blood serum sample of a pig specimen. Cat2 signal was employed for quantification of MT or total content of thiols.

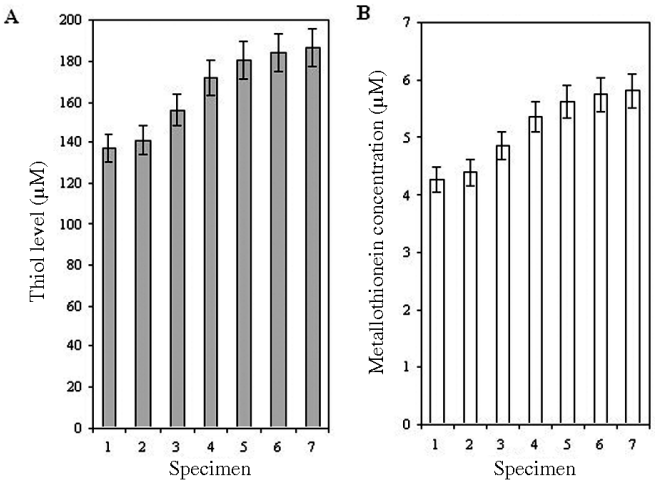

Fig. 3 Total content of thiols (A) and metallothionein (B) in blood serum sample of pig specimens (n = 7).

molecular thermostabile thiols) and content of MT. To determine total content of thiols we employed differential pulse voltammetry Brdicka reaction and measurements were carried out with automated electrochemical analyser (Fig. 1). The sample (5 or 10 µl) was injected to an electrochemical cell with 1,995 and/or 1,990 µl of the supporting electrolyte. The electrolyte and sample were cooled at 4 °C (PETRLOVA *et al.*, 2006). Then, the sample in the supporting electrolyte was measured under an accumulation time of 120 s. The voltammograms measured were smoothed and baseline corrected by GPES 4.9 software supplied by EcoChemie with respect to catalytic signal Cat2 (Fig. 2). Total content of thiols in blood serum samples of pig

specimens varied over the range from 137 to 186 μM. The average level of the thiols was estimated as 165 ± 20 μM (Fig. 3). The level of MT was determined by adsorptive transfer stripping technique coupled with differential pulse voltammetry Brdicka reaction. The sample (5 μl) was firstly accumulating on the surface of the working electrode for 120 s. Then the electrode was rinsed. The rinsed electrode was transferred to a pure supporting electrolyte containing cobalt (III) ions. The typical DP voltammetric curve is shown in Fig. 2. The MT level determined in the blood serum samples of the pigs' specimens was from 4.2 to 5.8 μM. The average MT level was 5.2 ± 0.6 μM (Fig. 3). From the animal species analysed to quantify MT level the highest level was determined in samples of *Gallus*, where the level exceeded 20 μM. A high level of MT was also observed in *Pogona vitticeps* and *Canis lupus lupus*. However the lowest level of MT of 3 μM was determined in *Trachemys scripta elegans, Budorcas taxicolor* and *Cervus elaphus* (ADAM et al., 2007).

CONCLUSION

It was shown that heat treatment of blood or blood serum is a very suitable purification step for the electrochemical determination of metallothionein. Based on the results obtained it can be concluded that this way of sample preparation is not only suitable for detection using voltammetry of MT but also for detection of low molecular mass thiols.

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