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Cyclic voltammetric study of the redox system of glutathione using the disulfide bond reductant tris(2-carboxyethyl)phosphine

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Abstract

The stabilization of the reduction state of proteins and peptides is very important for the monitoring of protein–protein, protein–DNA and protein–xenobiotic interactions. The reductive state of protein or peptide is characterized by the reactive sulfhydryl group. Glutathione in the reduced (GSH) and oxidized (GSSG) forms was studied by cyclic voltammetry. Tris(2-carboxyethyl)phosphine (TCEP) as the disulfide bond reductant and/or hydrogen peroxide as the sulfhydryl group oxidant were used. Cyclic voltammetry measurements, following the redox state of glutathione, were performed on a hanging mercury drop electrode (HMDE) in borate buffer (pH 9.2). It was shown that in aqueous solutions TCEP was able to reduce disulfide groups smoothly and quantitatively. The TCEP response at − 0.25 V vs. Ag/AgCl/3 M KCl did not disturb the signals of the thiol/disulfide redox couple. The origin of cathodic and anodic signals of GSH (at − 0.44 and − 0.37 V) and GSSG (at − 0.69 and − 0.40 V) glutathione forms is discussed. It was shown that the application of TCEP to the conservation of sulfhydryl groups in peptides and proteins can be useful instrument for the study of peptides and proteins redox behavior.

Keywords: Voltammetry; Hanging mercury drop electrode (HMDE); Glutathione (GSH, GSSG); Redox state; Sulfhydryl and disulfide groups; Tris(2-carboxyethyl)phosphine (TCEP); Hydrogen peroxide

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1. Introduction

The major part of proteins and peptides in natural conditions are present in reduced state [1–3]. Their redox states are intensively studied in connection with different scientific interests (e.g. free radicals and oxidative stress, protein–DNA binding, enzyme catalysis, cell cycle and cell death). The reductive state of protein or peptide molecule is often characterized by the reactive sulfhydryl group. One of such SH-containing peptides is glutathione. This ubiquitous tripeptide (L-γ-glutamyl-L-cysteinyl-glycine—Fig. 1A), discovered by F. G. Hopkins in 1921 [4], exists in living organisms in both reduced form (GSH) and oxidized form (GSSG). It is found that in most human tissues the GSH/GSSG ratio is more than 10/1 [5]. In cells, glutathione protects genetic materials playing an important role [5], in such reactions as (a) detoxification by binding to toxins (heavy metals, solvents, and pesticides), (b) antioxidation by scavenging reactive species (free radicals), and (c) synthesis of metabolizing enzymes which may be significant in cancer [6].

The oxidation/reduction of sulfhydryl/disulfide groups of proteins are based on electron transfer [7]. It is known that an organic disulfide (–SS–) can be reduced and/or sulfhydryl (–SH) oxidized according to the basic reaction scheme [8]:

$$R_1SSR_2 + (R_3SH)_n \leftrightarrows R_1SH + HSR_2 + R_3SSR_3 + (R_3SH)_{n-2} \quad (1)$$

Proteins might be oxidized during the sample preparation, purification and/or separation procedure. For the preservation of sulfhydryl groups thiol reductants such as 2-mercaptoethanol, dithiothreitol [9], thioglycolic acid and cysteine are obviously used [8]. Recently, for the reduction

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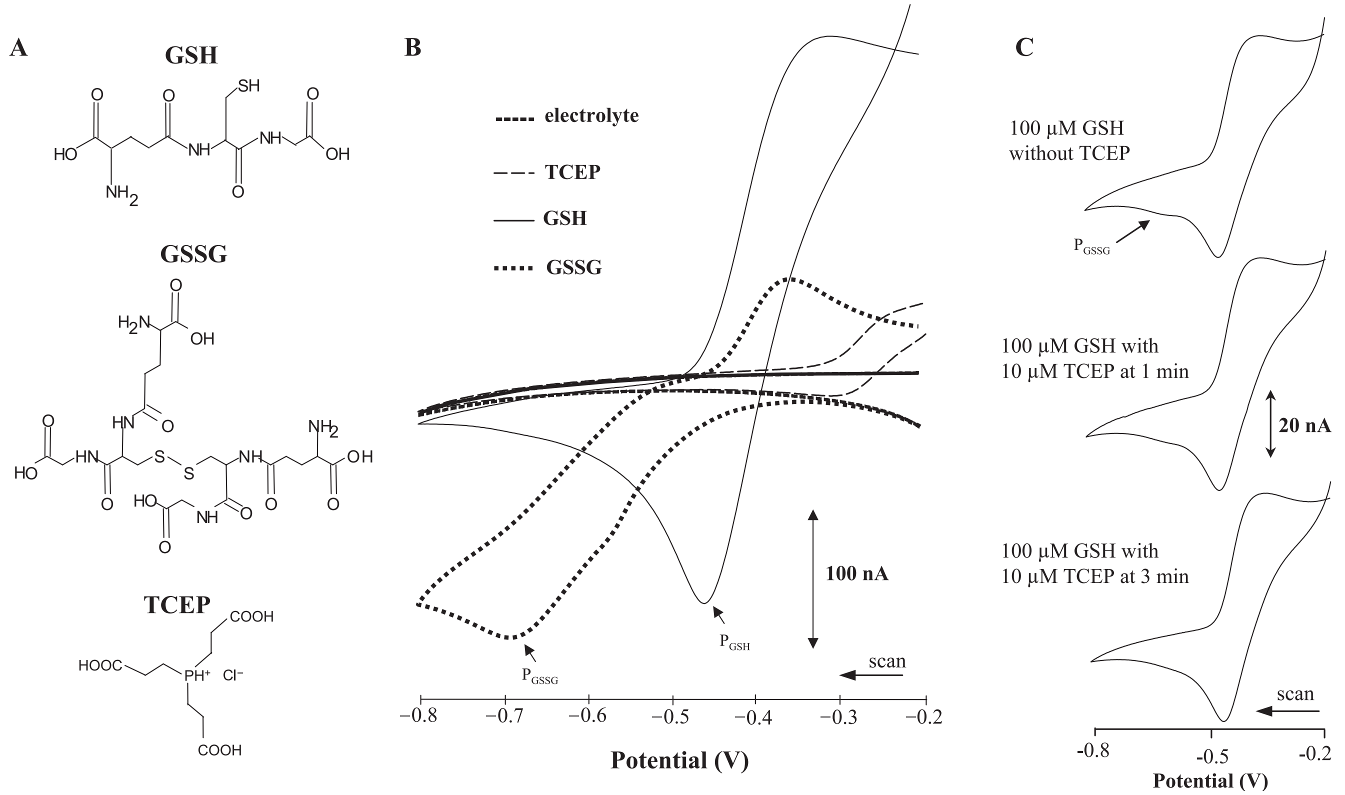

Fig. 1. (A) Chemical formulas of reduced (GSH) and oxidized (GSSG) forms of glutathione, and disulfide bond reductant: tris(2-carboxyethyl)phosphine (TCEP) as the chloride salt. (B) Cyclic voltammograms measured in 0.05 M borax ($Na_2B_4O_7$) at pH 9.2 for chemical compounds represented in A-part of this figure: 1 mM glutathione as GSH (solid-thin line), 0.5 mM GSSG (broken-thick line) and 0.25 mM of TCEP (broken-thin line). The buffer is shown as a solid-thick line. In the case of GSH measurement (P_{GSH}), 1.5 mM of TCEP for the reduction GSSG was applied. The arrows are showing cathodic peaks of GSH (P_{GSH}) and GSSG (P_{GSSG}). (C) Cyclic voltammograms of 100 µM GSH (a) without TCEP (b) and (c) with 10 µM TCEP after 1 and 3 min of pretreatment time (stirring of solutions at open circuit without any accumulation), respectively. Scan rate 25 mV/s, step potential 2 mV, vertex potential − 0.8 V, stirring 1420 rpm, for GSH measurements bubbling by argon 10 min. For other details see Experimental.

of disulfide bound tris(2-carboxyethyl)phosphine (TCEP) has been applied [10–12] (the chemical formula in Fig. 1A). In aqueous solutions, TCEP (e.g. L3P) reduces a disulfide group according to the reaction [8,10]:

$$L_3P + R_3SSR_3 + H_2O \rightarrow L_3PO + 2R_3SH \qquad (2)$$

where $L = CH_2CH_2COOH$. The strength of the phosphorus–oxygen bond renders the reduction irreversible.

The possibility to study proteins and peptides reduction and/or oxidation using electrochemical methods was presented in review [13]. For the determination of peptides and proteins containing sulfhydryl groups, catalytic processes proceeding at very negative potentials on mercury electrodes (Heyrovsky prenatrium wave [14], and Brdicka reaction [15]) were employed. High detection sensitivity has been found by using the adsorptive transfer stripping technique in combination with constant current chronopotentiometric analysis (AdTS CPSA)—peak H [16–20] and voltammetry in differential mode (DPV) in a cobalt(III)-containing solutions [21–24]. In these papers, it is claimed that during analyses the GSH/GSSG ratio is a very important experimental condition with respect to oxygen from the atmosphere, pH, buffers, and concentrations of components in solutions.

We describe the application of tris(2-carboxyethyl)phosphine (TCEP) for the controlled reduction of disulfide groups and of hydrogen peroxide for the oxidation of sulfhydryl groups in glutathione (GSH/GSSG). The effect of both reduction and oxidation agents on glutathione was followed by means of linear sweep cyclic voltammetry at a hanging mercury drop electrode (HMDE) polarized between potentials − 0.2 and − 0.8 V (vs. Ag/AgCl/3 M KCl).

2. Experimental

2.1. Apparatus

Experiments were performed with the AUTOLAB electrochemical instrument (EcoChemie, Utrecht, Netherlands) in connection with the VA-Stand 663 (Metrohm, Herisau, Switzerland). A three-electrode system was used including hanging mercury drop electrode (HMDE; surface area 0.4 mm^2) as a working electrode, Ag/AgCL/3 M KCl as a reference electrode, and Pt wire as an auxiliary electrode. The pH value was measured by Pracitronic (MV870, Germany).

2.2. Chemicals

In our experiments, reduced and oxidized glutathione (SigmaUltra purity, Sigma Aldrich) was used. Tris(2-carboxyethyl)phosphine (TCEP) is produced by Molecular Probes (Evgen, OR, USA). Other used chemicals were purchased from Sigma Aldrich. Bulk solutions were prepared before each electrochemical measurement. All solutions were prepared using deionized ACS water (Sigma Aldrich).

2.3. Procedure

Solutions, in which reduced form of glutathione was dissolved, were deoxygenated for 10 min by purging with water-saturated argon (99.99%) prior to measurements. Only experiments with oxidized form of glutathione were carried out in the presence of air oxygen. The cyclic voltammetry measurements were performed at HMDE in borate buffer (pH 9.2) and at room temperature. CV parameters were adjusted: start, vertex and end potential, − 0.2, − 0.8, − 0.2 V, respectively. The step potential was 2 mV, scan rate was 25 mV/s. The raw data were smoothed using level 4 of the Savitzky and Golay filter [25] of the GPES software (EcoChemie).

3. Results and discussion

Fifty years ago the electrochemical behavior of glutathione was described [26,27]. More recently, papers which include the application of voltammetric methods to the study of thiols and glutathiones have been published [28–34]. All studies describing the mechanism of the electrochemical reactions of glutathione at a mercury electrode draw substantially from Kolthoff's papers [26,27]. Our paper is aimed at the electrochemical study of the redox glutathione system: the reduction of disulfide bond in glutathione (GSSG) by the tris(2-carboxyethyl) phosphine (TCEP) and the oxidation of sulfhydryl group in glutathione (GSH) by hydrogen peroxide. Formulas of GSH, GSSG, and TCEP were shown in Fig. 1A.

First, we measured cyclic voltammograms of borate buffer, GSH, GSSG, and TCEP at an HMDE from the starting potential (− 0.2 V) to the vertex potential (− 0.8 V) and reverse to the initial potential. In Fig. 1B four cyclic voltammograms, which represent 0.05 M borax ($Na_2B_4O_7$), pH 9.2, 1 mM GSH, 0.5 mM GSSG and 0.25 mM TCEP are shown. The TCEP electrochemical signal (− 0.25 V vs. Ag/AgCl/3 M KCl) did not disturb responses of thiol/disulfide redox couple. The small TCEP signal at positive potentials is probably caused by the formation of an electrochemically active TCEP–mercury complex. This idea is supported by the fact that the TCEP, containing three carboxyl groups in the β-position to the phosphorus atom and having pK_a 2.99, 3.67, 4.36, and 7.66, remains in alkaline solutions (pH 9.2) negative charged anion (L3-) [35]. The anion has a large affinity to a positively charged mercury electrode. We observed cathodic and anodic signals not only for the oxidized form (GSSG), but also for reduced form (GSH) of glutathione. According to previous published papers [27,28,34] it was suggested that the cathodic signal of the oxidized form of glutathione (P_{GSSG} at − 0.69 V) corresponds to the GSSG reduction. We suppose that this reduction process ($GSSG + 2e^- + 2H^+ \rightarrow 2GSH$) is complicated by the follow-up formation of a compound of GSH with mercury, e.g. RS-mercury thiolate(s), which is oxidized during the anodic scan and produces a signal at about − 0.35 V.

Second, if the mercury electrode was polarized throughout the whole cycle in solutions containing only the reduced glutathione form (GSH), both cathodic (at − 0.45V) and anodic signals (at − 0.35 V) were observed. This can be explained by the formation of a reducible GS-mercury compound. Considering the pK_a values 3.59, 8.75, and 9.65 for GSH [36], it can be expected that glutathione is present in solutions (pH 9.2) not only in the zwitterionic form ($-NH_3^+$ and $-COO^-$) but also in the negatively charged form ($-NH_2$ and $-COO^-$). It can be supposed that the adsorption of negatively charged form of glutathione is strong at the positively charged electrode surface. The affinity of negative species and sulfur in glutathione molecules provide the possibility of producing compounds with mercury [28,34]. Therefore, the anodic signals, at about − 0.40 and − 0.35 V of GSSG and GSH, respectively, can correspond to the oxidation of slightly dissoluble mercurous GSHg(I) or mercuric GSHg(II) glutathionate formed according to the equations published by Jin et al. [34].

The cathodic GSSG voltammetric signal (P_{GSSG}) at − 0.69 V appeared as a good readable signal in solutions with air oxygen. It is interesting that in the case of the absence of oxygen the sharp peak (probably caused by adsorption/desorption properties on the electrode surface) at potentials about − 0.57 V was observed (not shown). A detailed study of the oxygen effect will be published elsewhere.

The effect of the TCEP on GSH behavior is illustrated in Fig. 1C. The 100 μM GSH was measured in solutions deoxygenated by argon purging (Fig. 1Ca). The another cyclic voltammograms were obtained after TCEP addition. The CV curves (Fig. 1Cb and c) were registered at waiting time of 1 and 3 min. In the case of (a) we were able to observe small signal of P_{GSSG} at potential − 0.69 V which disappeared after addition 10 mM TCEP (Fig. 1Cc). We concluded that the glutathione supplied by Sigma Aldrich (98% purity) can contain a small amount of oxidized form of glutathione.

We followed also the GSSG cathodic process in the presence of different TCEP concentrations (0.25–1.5 mM)—Fig. 2A. The thick curve (a) corresponds to 0.5 mM GSSG without TCEP. We could observe the well-developed P_{GSSG} cathodic signal without the P_{GSH} cathodic signal. The subsequent additions of TCEP to the solution of 0.5 mM GSSG caused an increase in the P_{GSH} signal (Fig. 2Ab–h). The reduction of GSSG by TCEP proceeds in aqueous solutions according to the equation:

$$(CH_2CH_2COOH)_3P + GSSG + H_2O \rightarrow (CH_2CH_2COOH)_3PO + 2GSH \qquad (3)$$

The changes of P_{GSH} and P_{GSSG} peak heights are shown in Fig. 2B. It is clear that peak P_{GSSG} decreases and peak P_{GSH} increases with the increasing of TCEP concentration. At TCEP concentration about 1.5 mM the signal P_{GSH} achieved maximum value while the P_{GSSG} signal disappeared (Fig. 2B). Similar results were obtained at 10 times lower concentrations of GSSG (not shown). According to the Eq. (3), for the total GSSG reduction is necessary the same TCEP molar concentration (the stechiometrical ratio 1:1). Higher consumption of TCEP than that expected only for the GSSG reduction was influenced by oxygen reduction.

In addition to the study of reduction process of glutathione the oxidation process of GSH using hydrogen peroxide was also studied. For this reason we investigated the CV responses of deoxygenated solutions containing 1 mM GSH and 0 mM, 0.6 mM and 0.9 mM hydrogen peroxide. We observed that the P_{GSH} peak

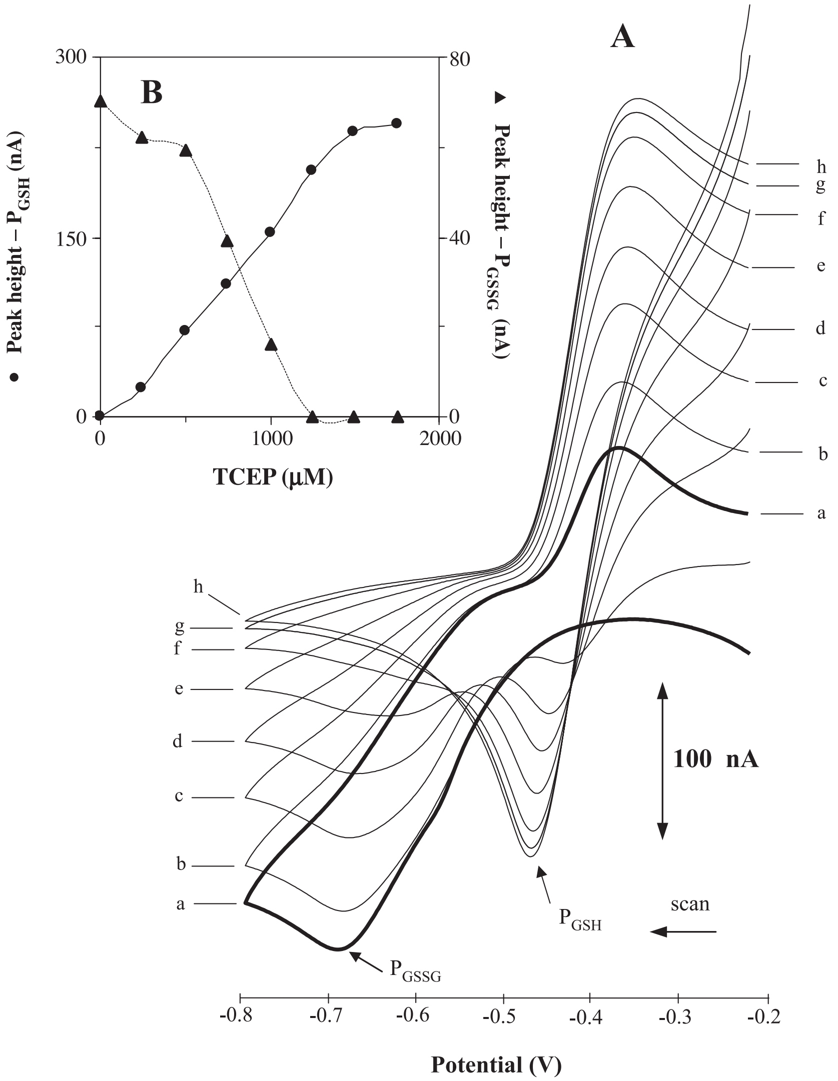

Fig. 2. (A) Cyclic voltammogram of (a) 0.5 mM GSSG marked as the thick line, and voltammograms recorded after addition of increasing TCEP concentration to GSSG, marked as thin lines. The increase in TCEP concentration was realized by addition of 0.25 mM (from 0 to 1.5 mM) between individual recordings of the electrochemical curves (voltammograms b to h). Pretreatment time after an individual addition of TCEP was 2 min. Dependences of peak P_{GSH} and P_{GSSG} heights (B) on TCEP concentration. Other conditions as in Fig. 1.

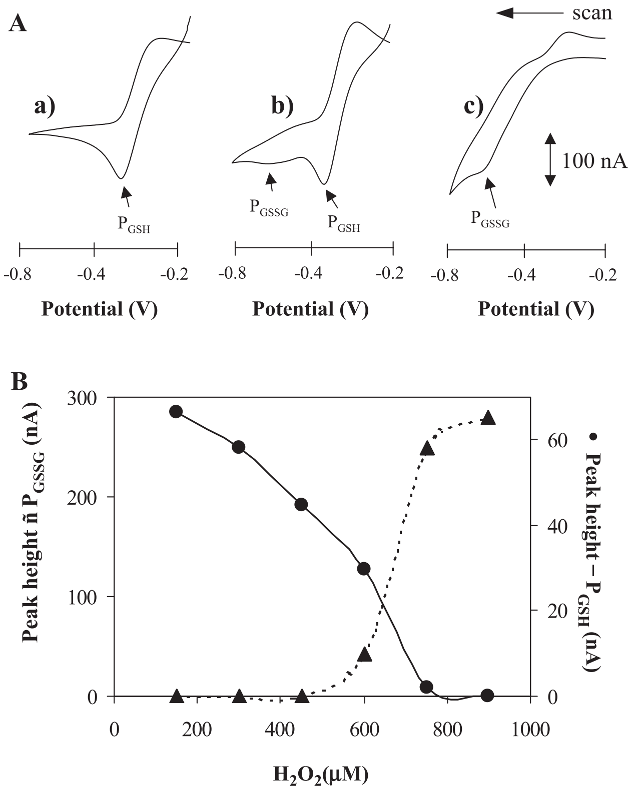

Fig. 3. (A) Cyclic voltammograms of 1 mM GSH recorded after two additions of hydrogen peroxide: (a) 0 mM (total reduced glutathione), (b) 0.6 mM (partly oxidized glutathione), and (c) 0.9 mM (total oxidized glutathione). (B) Dependence of P_{GSH} and P_{GSSG} peak heights on peroxide concentration. Other conditions as in Fig. 1.

height decreased with increasing H_2O_2 concentration. On the other hand, the P_{GSSG} peak height was increased with the addition of H_2O_2. The results obtained are summarized in Fig. 3A and B. We observed that the ratio of the thiol/disulfide forms could be determined from the plots of GSH/GSSG peak heights vs. the concentration of reducing (TCEP) or oxidizing (H_2O_2) reagent (Fig. 3B).

This communication shows that besides the two common thiol reductants (2-mercaptoethanol and dithiothreitol), used for the preservation of sulfhydryls groups, tris(2-carboxyethyl)phosphine (TCEP) can also be used. From a bioanalytical point of view the TCEP is suitable as a reduction agent for the electrochemical detection of the glutathione redox state. Moreover, we believe that this system can be applicable for the study of redox processes of peptides and proteins.

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References

[1] D.A. Dickinson, H.J. Forman, Cellular glutathione and thiols metabolism, Biochem. Pharmacol. 64 (2002) 1019–1026.

[2] S. Carelli, A. Ceriotti, A. Cabibbo, G. Fassina, M. Ruvo, R. Sitia, Cysteine and glutathione secretion in response to protein disulfide bond formation in the ER, Science 277 (1997) 1681–1684.

[3] F.Q. Schafer, G.R. Buettner, Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple, Free Radic. Biol. Med. 30 (2001) 1191–1212.

[4] F.G. Hopkins, Biochem. J. 15 (1921) 286–305.

[5] B. Halliwell, J.M.C. Gutteridge, Free Radicals in Biology and Medicine, 3rd ed., Oxford Univ. Press, New York, 1993, pp. 140–153.

[6] A. Meister, M.E. Anderson, Glutathione, Ann. Rev. Biochem. 52 (1983) 711–760.

[7] P.C. Jocelyn, Chemical-reduction of disulfides, Methods Enzymol. 143 (1987) 246–256.

[8] U.T. Rüeg, J. Rudinger, Reductive cleavage of cystine disulfides with tributhylphosphine, Methods Enzymol. 47 (1977) 111–126.

[9] W.W. Cleland, Dithiothreitol, a new protective reagent for SH groups, Biochemistry 3 (1964) 480–482.

[10] J.A. Burns, J.C. Butler, J. Moran, G.M. Whitesides, Selective reduction of disulfides by tris(2-carboxyethyl)phosphine, J. Org. Chem. 56 (1991) 2648–2650.

[11] A. Tiwari, L.J. Hayward, Familiar amyotrophic lateral sclerosis

mutants of copper/zinc superoxide dismutase are susceptible to disulfide reduction, J. Biol. Chem. 278 (2003) 5984–5992.

[12] J. Lykkesfeldt, Determination of ascorbic acid and dehydroascorbic acid in biological samples by high-performance liquid chromatography using subtraction methods: reliable reduction with tris[2-carboxyethyl]phosphine hydrochloride, Anal. Biochem. 282 (2000) 89–93.

[13] M.J. Honeychurch, The reduction of disulfide bonds in proteins at mercury electrodes, Bioelectrochem. Bioenerg. 44 (1997) 13–21.

[14] J. Heyrovsky, J. Kuta, Principles of Polarography, Czech Acad. Sci, Prague, 1965.

[15] R. Brdicka, Polarographic studies with the dropping mercury cathode: Part XXXI. A new test for proteins in the presence of cobalt salts in ammoniacal solutions of ammonium chloride, Collect. Czechoslov. Chem. Commun. 5 (1933) 112–128.

[16] M. Tomschik, L. Havran, M. Fojta, E. Palecek, Constant current chronopotentiometric stripping analysis of bioactive peptides at mercury and carbon electrodes, Electroanalysis 10 (1998) 403–409.

[17] M. Tomschik, L. Havran, E. Palecek, M. Heyrovsky, The presodium catalysis of electroreduction of hydrogen ions on mercury electrodes by metallohionein. An investigation by constant current derivative stripping chronopotentiometry, Electroanalysis 12 (2000) 274–279.

[18] R. Kizek, L. Trnkova, E. Palecek, Determination of metallothionein at the femtomole level by constant current stripping chronopotentiometry, Anal. Chem. 73 (2001) 4801–4807.

[19] L. Trnkova, R. Kizek, J. Vacek, Catalytic signal of rabbit liver metallothionein on a mercury electrode: combination of derivative chronopotentiometry with adsorptive transfer stripping, Bioelectrochemistry 56 (2002) 57–61.

[20] M. Strouhal, R. Kizek, J. Vacek, L. Trnkova, M. Nemec, Electrochemical study of heavy metals and metallothionein in yeast Yarrowia lipolytica, Bioelectrochemistry 60 (2003) 29–36.

[21] B. Raspor, Elucidation of the mechanism of the Brdicka reaction, J. Electroanal. Chem. 503 (2001) 159–162.

[22] B. Raspor, M. Paic, M. Erk, Analysis of metallothionein by the modified Brdicka procedure, Talanta 55 (2001) 109–115.

[23] J. Vacek, J. Petrek, R. Kizek, L. Havel, B. Klejdus, L. Trnkova, F. Jelen, Electrochemical determination of lead and glutathione in a plant cell culture, Bioelectrochemistry 63 (2004) 347–351.

[24] M. Erk, D. Ivankovic, B. Raspor, J. Pavicic, Evaluation of different purification procedures for the electrochemical quantification of mussel metallothioneins, Talanta 57 (2002) 1211–1218.

[25] M.U.A. Bromba, H. Ziegler, Application hints for Savitzky–Golay digital smoothing filters, Anal. Chem. 53 (1981) 1583–1586.

[26] I.M. Kolthoff, W. Stricks, N. Tanaka, The polarographic prewaves of cysteine (RSSR) and dithioglycolic acid (TSST) and the oxidation potentials of the systems RSSR-RSH and TSST-TSH, J. Am. Chem. Soc. 77 (1955) 4739–4742.

[27] W. Stricks, I.M. Kolthoff, Polarography of glutathione, J. Am. Chem. Soc. 74 (1952) 4646–4653.

[28] M. Heyrovsky, P. Mader, S. Vavricka, V. Vesela, M. Fedurco, The anodic reaction at mercury electrodes due to cystein, J. Electroanal. Chem. 430 (1997) 103–117.

[29] M. Heyrovsky, S. Vavricka, Electrochemical reactivity of homocysteine at mercury electrodes as compared with cysteine, Bioelectrochem. Bioenerg. 48 (1999) 43–51.

[30] C. Harlyk, G. Bordin, O. Nieto, A.R. Rodriguez, Electrochemical behaviour of a metallothionein related peptide in the presence of cadmium using cyclic voltammetry, J. Electroanal. Chem. 446 (1998) 139–150.

[31] C. Harlyk, O. Nieto, G. Bordin, A.R. Rodriguez, Electrochemical study of metallothioneins using cyclic voltammetry, J. Electroanal. Chem. 458 (1998) 199–208.

[32] E.I. Korotkova, Y.A. Karbainov, A.V. Shevchuk, Study of antioxidant properties by voltammetry, J. Electroanal. Chem. 518 (2002) 56–60.

[33] V.D. Parker, L.C. Seefeldt, A mediated thin-layer voltammetry method for the study of redox protein electrochemistry, Anal. Biochem. 247 (1997) 152–157.

[34] W.R. Jin, X. Zhao, L. Xiao, Mechanism of the electrochemical reaction of glutathione at a mercury electrode, Electroanalysis 12 (2000) 858–862.

[35] J. Podlaha, J. Podlahova, Compounds structurally related to complexone: I. Tris(carboxyethyl)phosphine, Collect. Czechoslov. Chem. Commun. 38 (1972) 1730–1736.

[36] J. Bjerrum, Stability Constants, Chemical Society, London, 1958.