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Femtogram Electroanalytical Detection of Prostatic Specific Antigen by Brdicka Reaction

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Prostatic-specific antigen is considered as the best marker for prostate cancer. Due to the importance of PSA for diagnostic purposes it is not surprising that there are tested and optimized various methods for its determination. In spite of such intensive research in the field of electrochemical detection of some by-products connected with concentration of PSA, electrochemical behaviour of PSA has not been studied yet. The aim of this study was to investigate electrochemical catalytic signals of PSA using differential pulse voltammetry Brdicka reaction. The catalytic signals were studied using adsorptive transfer stripping technique as well as directly in the electrochemical cell. Nevertheless, we primarily tested detection of PSA by standard immunoanalysis and by gel and capillary chip electrophoresis to investigate behaviour of this protein in electric field. Both electrophoretic methods showed that the most intensive band of PSA was determined at 37 kDa under reducing conditions and at 26 kDa under non-reducing. Band at 37 kDa corresponds to a reduced, and at 26 kDa to non-reduced PSA. Studying of basic electrochemical behaviour of PSA was primarily carried out using standard electrochemical cell and HMDE as a working electrode. $Co(NH_3)_6Cl_3$ (1 mM) was used as a supporting electrolyte. Temperature of the electrolyte was maintained at 4 °C. The effects of accumulation time and

concentration of $Co(NH_3)_6Cl_3$ as a key component of supporting electrolyte were studied. Time of accumulation of 240 s and 1.00 mM $Co(NH_3)_6Cl_3$ were found the optimal for detection of PSA in electrochemical cell. Further, we used adsorptive transfer stripping technique coupled with differential pulse voltammetry Brdicka reaction for detection of PSA. Two temperatures of adsorption as 4 °C and 20 °C and several times of adsorption as 40, 60, 80, 120, 180, 240, 360 and 420 s were tested. Under the optimized conditions (4 °C temperature of adsorption and 240 s time of adsorption) calibration curve with the following equation y = 36.865x - 11.949, R^2 = 0.9911 within the concentration interval from 1 to 1,500 pg/ml was measured. Detection limit of PSA expressed as 3 S/N was estimated down to 1 fg of PSA in 0.5 μl.

Keywords: prostate specific antigen; electrochemical detection; electrophoresis; voltammetry; adsorptive transfer stripping technique; catalytic signal; protein

1. INTRODUCTION

1.1 Prostate specific antigen

Prostatic-specific antigen (short name PSA and alternative name: gamma-seminoprotein, kallikretin-3, P-30 antigen and semenoglease) is a serine protease (EC 3.4.21.77) secreted by both normal prostate glandular cells and prostate cancer cells [1]. Structure of this protein is shown in Fig. 1A. Biological function of PSA is lyses the coagulum formed upon ejaculation when prostatic fluid is mixed with seminal vesicle fluids [2]. From the point of view of primary structure, PSA is a single chain glycoprotein of 237 amino acid residues, 4 carbohydrate side chains and multiple disulphide bonds [2]. Based on the amino acids moieties composition leucine, valine and glycine belong to the most abundant, but content of cysteine is also considerable (Fig. 1B). PSA occurs in two forms as bound to α-1-antichymotrypsin and free often abbreviated with f-PSA. The total amount of bound PSA and f-PSA is known as total PSA (t-PSA). An increasing of t-PSA level at more that 4 ng/ml represents strong indicators of the possibility of prostate cancer, which is the most common cancer in men and is the second leading cause of cancer death in men worldwide [3]. However, elevated serum PSA levels have also been attributed to benign prostatic hyperplasia. In spite of this fact PSA is the best tumour marker used in oncology [4-6]. Moreover, there attempts to find differences between prostate carcinoma and benign prostatic hyperplasia as determination of the f-PSA/t-PSA ratio [7]. Early diagnostics of prostate carcinoma is crucial for the successful treatment of this disease [8-11].

1.2 Analytical detection of PSA

Due to the importance of PSA for diagnostic purposes it is not surprising that there are tested and optimized various methods for its determination. Enzyme-linked immunosorbent assays (ELISA), fluorescence-based immunoassays and electrochemiluminenesence-based immunoassays belong to the most commonly methods for this purpose [12]. These methods have good sensitivity and specificity,

but these demand on laboratory equipment. Based on this, sensors and biosensors are of great interest for determination of PSA [13].

1.2.1 Immunosensors

Electrochemical based sensors and biosensors are based on indirect detection of PSA. Sandwich immunoassays with electrochemical detection of antibody labelled with an enzyme subsequently cleaving substrate generating electroactive molecules as. alkaline phosphatase [14], horseradish peroxidase [15-18], β-galactosidase [19-21], and/or glucose oxidase [22] belong to often used methods. There was also suggested biosensor for detection of PSA enzyme activity based on the using the ferrocene functionalized peptide probes. The measurement is based on the specific proteolytic cleavage of the peptide on the electrode surface in the presence of PSA, resulting the change of the current signal of the electrode [23].

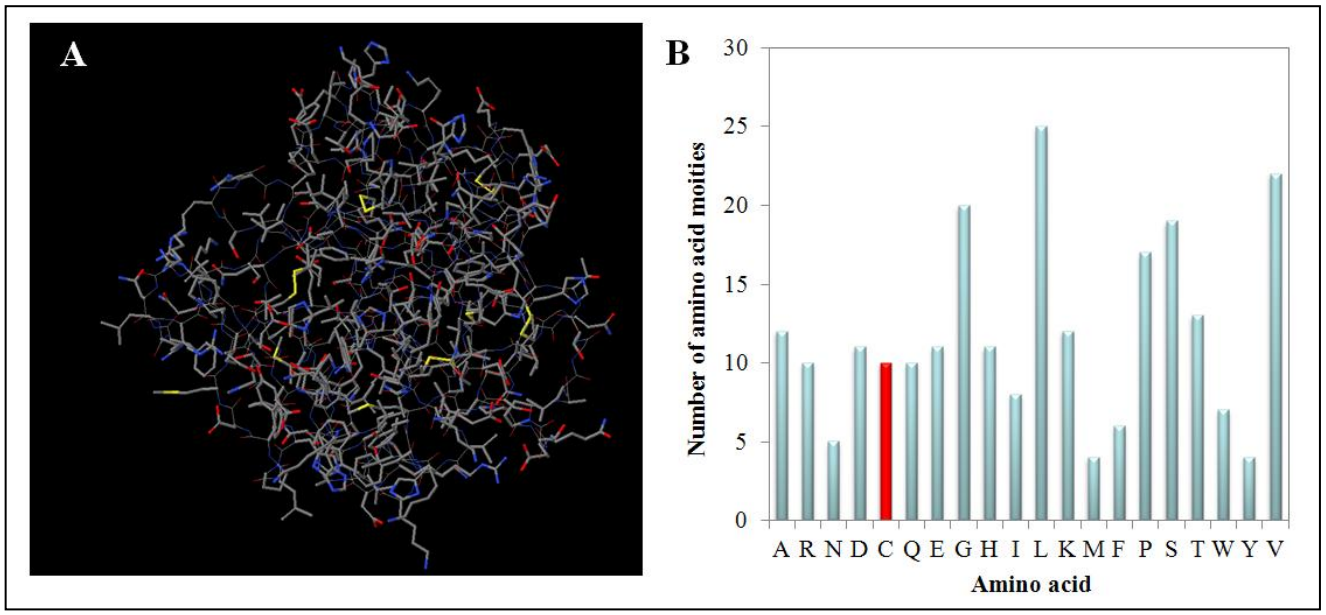

Figure 1. (**A**) Structure of PSA modified according to database http://www.ebi.ac.uk/pdbsum/1PFA. (**B**) Amino acid composition of PSA as a single chain glycoprotein of 237 amino acid residues [2].

1.2.2 Nanotechnologies in PSA detection

Nanotechnology-based materials are very convenient for fabrication of sensors and biosensors [24]. Detecting of PSA labelled with quantum dots (QDs) [25], or with quantum dot functionalized graphene sheets as labels [26], or immunochromatographic strip technique [27] reached detection limits similar to those obtained by immune-based methods. There was also suggested sensor based on binding of PSA to gold particle with detection limit of PSA as units of pg/ml. The sensor measured changes on the surface of the electrode [28]. Dumbbell-like Au-Fe(3)O(4) nanoparticles as label for

the preparation of electrochemical immunosensors were also successfully used for detection of PSA [29], Sensitive electrochemical immunosensor for PSA with signal enhancement based on nitrodopamine-functionalized iron oxide nanoparticles [30] and amperometric immunosensor for PSA based on gold nanoparticles/ionic liquid/chitosan hybrid film [31] were suggested. Moreover, a direct electrochemical immunoassay format was employed to detect PSA based on the potential change before and after the antigen-antibody interaction [15,32].

1.2.3 Detection of PSA via interaction with metal ions

Interaction of PSA with metal ions resulting in biocatalytic metal deposition on electrode surface, especially of silver [33,34] or gold [35] origin, represent other group electrochemical biosensors for detection of this protein. Detection of PSA via interactions of this protein with other metal particles is summarized and discussed in the paper of Sarkar et ak.[36]. In addition, there was suggested surface plasmon resonance sensor using colloidal gold and latex microparticles. Detection limit of this method was 0.15 ng/ml PSA [37]. Towards fully automated procedures there are developed simpler approaches, including amperometric detectors, in which it was possible to detect PSA at a concentration around the units ng/ml [32,38].

1.2.4 Microfluidic techniques

Microfluidic and chip techniques coupled with nanoparticles based isolation and electrochemical detection are used for multiplex immunosensors for PSA detection. This topic was discussed in the paper of Henares et al. [39]. There was suggested microfluidic-based affinity assay device for running bead-protocols. This innovative system combines a special chip containing eight polymer microchannels, with a portable, computer-controlled instrument. Both immunosensing strategies developed promise to be a sensitive, multiplexed tool for fast and easy PSA analysis [40]. Other authors tested integrated microfluidic systems with an immunosensor modified with carbon nanotubes for detection PSA in human serum samples [38]. The application was successful, which is confirmed in the following papers as microfluidic chip-based nanoelectrode array [35], electrochemical multiplexed assay [40], microchip-based multiplex electro-immunosensing system [41] and microfluidic electrochemical immunoarray [42].

1.2.5 Label free techniques

Nanoporous gold film based immunosensor for label-free detection of PSA was also suggested. The sensing signal is based on the monitoring of the electrode's current response towards $K_3Fe(CN)_6$, which is extremely sensitive to the formation of immunocomplex within the nanoporous film [43]. Label-free immunosensor for PSA based on single-walled carbon nanotube array-modified microelectrodes was prepared and tested. The current signals, derived from the oxidation of tyrosine (Tyr), and tryptophan (Trp) residues, increased with the interaction between t-PSA on t-PSA-mAb

covalently immobilized on single walled nanotubes [44]. In spite of such intensive research in the field of electrochemical detection of some by-products connected with concentration of PSA, electrochemical behaviour of PSA has not been studied yet. The aim of this study was to investigate electrochemical catalytic signals of PSA using Brdicka reaction. The catalytic signals were studied using adsorptive transfer stripping technique as well as directly in the electrochemical cell.

2. EXPERIMENTAL PART

2.1 Chemicals

Tris(2-carboxyethyl)phosphine (TCEP) was from Molecular Probes (USA). PSA and other chemicals used in this study were purchased from Sigma Aldrich (USA). PSA stock standard solutions were prepared with ACS water (Sigma-Aldrich) and stored in the dark at –20 °C. Working standard solutions were prepared daily by dilution of the stock solution. To pipette volumes down to micro and nanolitres, used pipettes were purchased from Eppendorf Research (Germany) with the highest certified deviation (±12 %).

2.2 Differential pulse voltammetry Brdicka reaction – Electrochemical cell

Differential pulse voltammetric measurements were performed with 747 VA Stand instrument connected to 746 VA Trace Analyzer and 695 Autosampler (Metrohm, Switzerland), using a standard cell with three electrodes and cooled sample holder (4 °C). A hanging mercury drop electrode (HMDE) with a drop area of 0.4 and/or 0.25 mm^2 was the working electrode. An Ag/AgCl/3M KCl electrode was the reference and glassy carbon electrode was auxiliary. For data processing, GPES 4.9 supplied by EcoChemie was employed. The analysed samples were deoxygenated prior to measurements by purging with argon (99.999 %), saturated with water for 120 s. Brdicka supporting electrolyte containing 1 mM $Co(NH_3)_6Cl_3$ and 1 M ammonia buffer ($NH_3(aq)$ + NH_4Cl, pH = 9.6) was used. The supporting electrolyte was exchanged after each analysis. The parameters of the measurement 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, volume of injected sample: 20 µl (100 × diluted sample with 0.1 M phosphate buffer pH 7.0). All experiments were carried out at temperature 4 °C employing thermostat Julabo F25 (Labortechnik GmbH, Germany) [45].

2.3 Differential pulse voltammetry Brdicka reaction – Adsorptive transfer stripping technique

The principle of the adsorptive transfer stripping technique (AdTS) is based on the strong adsorption of the target molecule on the surface of the working electrode at an open circuit. The hanging mercury drop electrode is periodically renewed. Target molecules are adsorbed on the surface of the renewed working electrode at an open circuit. The electrode is washed with a supporting

electrolyte. The electrode with the adsorbed target molecules is measured in the presence of the supporting electrolyte. PSA 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) was 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 [46,47].

2.4 Capillary electrophoresis

Analyses on an automated microfluidic Experion electrophoresis system (Bio-Rad, USA) were carried out according to the manufacturer's instructions with supplied chemicals (Experion Pro260 analysis kit, Bio-Rad). A sample (4 µl) was mixed either with 2 µl of non-reducing sample buffer or 2 µl of reducing sample buffer (30 µl of nonreducing sample buffer and 1 µl β-mercaptoethanol, and after 4 min boiling, 84 µl of water was added. After the priming of the chip with the gel and gel-staining solution in the diluted priming station sample, the mixture (6 µl) was loaded into the sample wells. The Pro260 Ladder included in the kit was used as a standard. For operation and standard data analysis Experion software version 3.10 (Bio-Rad, USA) was used [48,49].

2.5 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The electrophoresis was performed according to Laemmli [50] using a Mini Protean Tetra apparatus with gel dimension of 8.3 × 7.3 cm (Bio-Rad, USA). First 12.5 % (*m/V*) running, then 5 % (*m/V*) stacking gel was poured. The experimental conditions were optimized by Krizkova et al. [48,49]. Briefly, the gels were prepared from 30 % (*m/V*) acrylamide stock solution with 1 % (*m/V*) bisacrylamide. The polymerization of the running or stacking gels was carried out at room temperature for 45 min or 30 min, respectively. Prior to analysis, the samples were mixed with non-reduction sample buffer in a 2:1 ratio. The samples were boiled for 2 min, and then 4 µl of the sample was loaded onto a gel. For determination of the molecular mass, the protein ladder "Precision plus protein standards" from Biorad was used. The electrophoresis was run at 150 V for 1 h (Power Basic, Biorad USA) in tris-glycine buffer (0.025 M Trizma-base, 0.19 M glycine and 3.5 mM SDS, pH = 8.3). Silver staining of the gels was performed according to Oakley et al. [51].

2.6 Automatic immunoanalysis of t-PSA

Total PSA (t-PSA) was determined by immunochemical analyzer AIA 600 II (Tosoh Corporation, Japan). AIA 600 II is specifically designed for measurement of immunochemical parameters in biological fluids using reagents of AIA-PACK series. Analyses were carried out according to the manufacturer's instructions. The instrument was calibrated using AIA-PACK Calibrator set using 6 point calibration (Tosoh Corporation, Japan). All reactions were performed in

the special disposable test cups containing dried and lyophilized reagents. Immunochemical antigen-antibody reaction employed magnetic particles (1.5 mm). Samples were incubated at 37ºC. 4-methylumbelliferyl phosphate was used as a substrate and fluorescence corresponding to enzymatic activity on magnetic particles was measured.

2.7 Preparation of deionised water and pH measurement

The deionised water was prepared using reverse osmosis equipment Aqual 25 (Czech Republic). The deionised water was further purified by using apparatus MiliQ Direct QUV equipped with the UV lamp. The resistance was 18 MΩ. The pH was measured using pH meter WTW inoLab (Weilheim, Germany).

2.8 Mathematical treatment of data and estimation of detection limits

Mathematical analysis of the data and their graphical interpretation was realized by software Matlab (version 7.11.). Results are expressed as mean ± standard deviation (S.D.) unless noted otherwise (EXCEL®). The detection limits (3 signal/noise, S/N) were calculated according to Long and Winefordner [52], whereas N was expressed as standard deviation of noise determined in the signal domain unless stated otherwise.

3. RESULTS AND DISCUSSION

3.1 Gel electrophoresis of PSA

Immunochemical methods based on fluorimetric detection and magnetic particles bearing antibodies based isolation are commonly used for PSA detection in clinical practise [20,21]. Therefore, we primarily used the automatic assay based on that in this study. Typical calibration dependence measured on this instrument is shown in Fig. 2A. The dependence was linear within the concentration interval from 1 to 100 ng/ml. Relative standard deviation was lower than 1 % (n = 5). It is obvious that this method is robust. However, there is no space for studying of changes in the protein, structural alterations, polymerization etc. SDS-PAGE is an excellent tool how to study polymerization of the proteins [53,54]. Analysis of PSA using SDS-PAGE showed that protein structure with molecular mass app. 35 kDa was formed under reducing conditions (Fig. 2B, left) and protein with molecular mass app. 25 kDa under non-reducing conditions (Fig. 2B, right). Higher molecular structure detected under reducing conditions can be related to the fact that PSA sub-units could form higher molecular mass complexes due to reduced –SH moieties. Moreover, PSA as glycoprotein behave atypically in electrophoresis and can form other types of complexes [55]. From the point of view of sensitivity, we were able to detect 8.7 ng of PSA under reducing conditions, but PSA of 17.5 ng was nod detected under non-reducing ones.

3.2 Capillary electrophoresis PSA

Capillary chip electrophoresis is an automated microfluidic electrophoresis system that uses a Caliper Life Sciences innovative LabChip microfluidic separation technology with sensitive fluorescent sample detection. It performs rapid and repeatable analyses of protein, DNA and RNA samples, which allows the analysis of protein or nucleic acids samples within 30 min. The separation, detection and data analysis are performed within a single platform, so the time-consuming steps in classic electrophoretic methods are minimized.

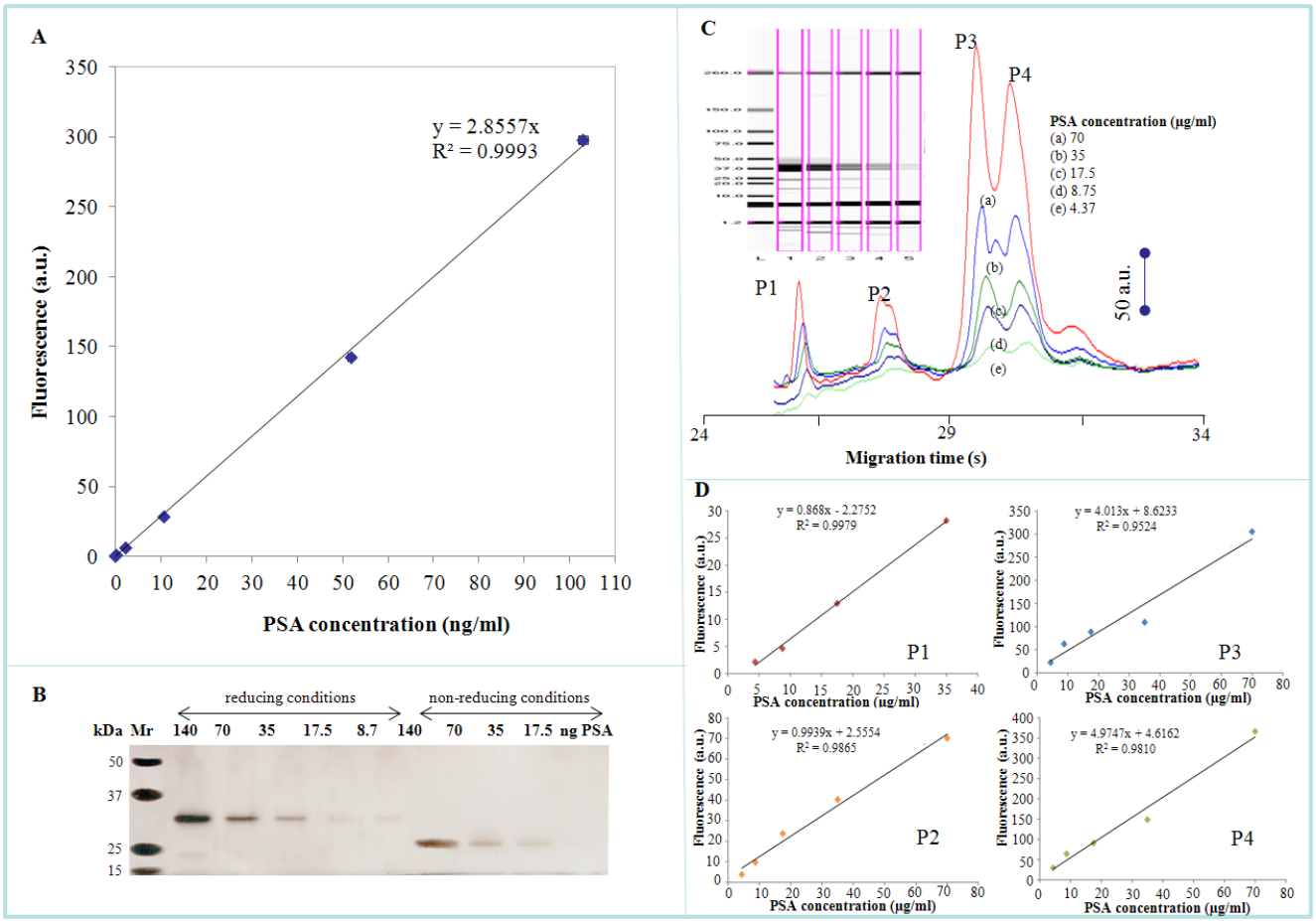

Figure 2. Immunochemical detection. (**A**) Dependence of fluorescence signal of concentration of PSA within the concentration interval from 1 to 100 ng/ml. 4-methylumbelliferyl phosphate was used as a substrate. (**B**) Polyacrylamid gel electrophoresis of PSA (8.7, 17.5, 35, 70 and 140 ng) reducing and non-reducing conditions. The electrophoresis was run at 150 V for 1 h in tris-glycine buffer. Gels were stained with silver. (**C**) Capillary chip electrophoresis of PSA (4.37, 8.75, 17.5, 35 and 70 ng). Sample of 6 µl was loaded ; in inset: virtual gel based on the chip analysis shown below. (**D**) Dependencies of peaks P1, P2, P3 and P4 on PSA concentration. For other experimental conditions see in Experimental section.

Many types of samples, such as bacterial lysates, protein extracts, and chromatography fractions, can be analysed. In addition to the significant shortening of time required, the chip-based

method allows both repeatable and accurate sizing and quantification of the proteins [53,54,56-59]. In the following part of this study, capillary chip electrophoresis was used for analysis of PSA, because this method is able to analyse various protein polymeric structures and quantify them. . To our knowledge, this system has not been previously used for PSA analysis. Therefore, unique results of chip capillary electrophoretic analysis of PSA are shown Figs. 2C and D. We found that the most intensive band on the virtual gel shown in inset in Fig. 2C was determined at 37 kDa under reducing conditions and at 26 kDa under non-reducing. These results well corresponds with the above-mentioned results as well as with the previously published results [55]. Band at 37 kDa corresponds to a reduced, and at 26 kDa to non-reduced PSA. Band of molecular mass of 15 kDa can be considered as a product of PSA auto-proteolytic reaction. Glycosylation of PSA is the most likely explanation for atypical migration of this protein. Places, in which PSA is glycosylated, or other interactions with proteins and other biomolecules significantly affect the mobility of PSA [1,55]. In addition, it is known that PSA is glycosylated differently and different isoforms differ in glycosylation. On the other hand, PSA glycosylation can be used for diagnosing prostate cancer [60-63].

Because capillary chip system is able to provide also capillary electrophoresis, we were able to determine electrophoretic profile of PSA, which is shown in Fig. 2C. During PSA analysis, there were identified four electrophoretic peaks marked as P1 (migration time "MT" = 26 s), P2 (MT = 27.5 s), P3 (MT = 31 s) and P4 (MT 32 s). Majority P3 and P4 signals correspond to the predominant protein structure PSA. Detailed study of electrophoretic behaviour of these peaks and their origin will be published elsewhere. Moreover, we found that all identified electrophoretic signals depend on the concentration of PSA (analysis performed in the concentration range of 4.37 μg/ml to 70 μg/ml, Fig. 2D). Peak P1 was strictly linear as showing the calibration dependence with the following equation $y = 0.868x - 2.2752$, $R^2 = 0.9979$, Peaks P2 and P4 linearly enhanced with the increasing concentration of PSA according to the following equations $y = 0.9939x + 2.5554$, $R^2 = 0.9865$, and $y = 4.9747x + 4.6162$, $R^2 = 0.9810$. Dependence of Peak P3 height on concentration of PSA was expressed as $y = 4.013x + 8.6233$ with the lowest $R^2 = 0.9524$.

3.3 Electrochemical analysis PSA

Electrochemical behaviour of PSA has not been studied yet to our knowledge and most of electrochemical biosensors are based on immunosensing and some enzyme based reaction [28,30,41,44]. More than eighty years ago Brdicka and Heyrovsky discovered electrochemical catalytic signals of proteins on mercury electrode in the presence of Co(III) ammonia complex, which have been then intensively studied [64-70]. These very interesting signals called Brdicka reaction are associated with a catalytic hydrogen evolution from the supporting electrolyte due to the presence of active protein moieties, mainly, due to -SH ones [47,71-75]. In this area, our attention is aimed at the low molecular mass protein called metallothionein, which provides well characterized Brdicka catalytic signals and these signals can be used for determination of this protein in various types of samples including blood serum of patients with cancer [45,47,76-86]. Catalytic hydrogen evolution due to the presence of PSA has not yet been studied, although the PSA molecule with free-SH groups

(Fig. 1). Studying of basic electrochemical behaviour of PSA was primarily carried out using standard electrochemical cell and HMDE as a working electrode. $Co(NH_3)_6Cl_3$ (1 mM) was used as a supporting electrolyte. Temperature of the electrolyte was maintained at 4 °C, which was previously found as the best from the point of view of low relative standard deviation and high sensitivity for detection of various proteins [46,47]. Typical DP voltammograms of PSA (5 ng/ml) are shown in Fig. 3A. The effect of various times of accumulation of PSA on the electrode surface is obvious. PSA was accumulated at various times of accumulation as 30, 60, 90, 120, 180, 240, 360 and 420 s at open circuit. Determined signals RS2Co, Cat1 and Cat2 changed markedly with the increasing time of accumulation. Poor delectable RS2Co increased from 120 s long accumulation (Fig. 3B). The peak was of sharp shape between 180 to 240 s long accumulations. Peak Cat1 enhanced slowly with the increasing accumulation time and, at accumulation time of 240 s, this peak was beginning to overlap with RS2Co. Catalytic Cat2 gradually enhanced with the increasing time of accumulation and reached a maximum at 240 s (Fig. 3C).

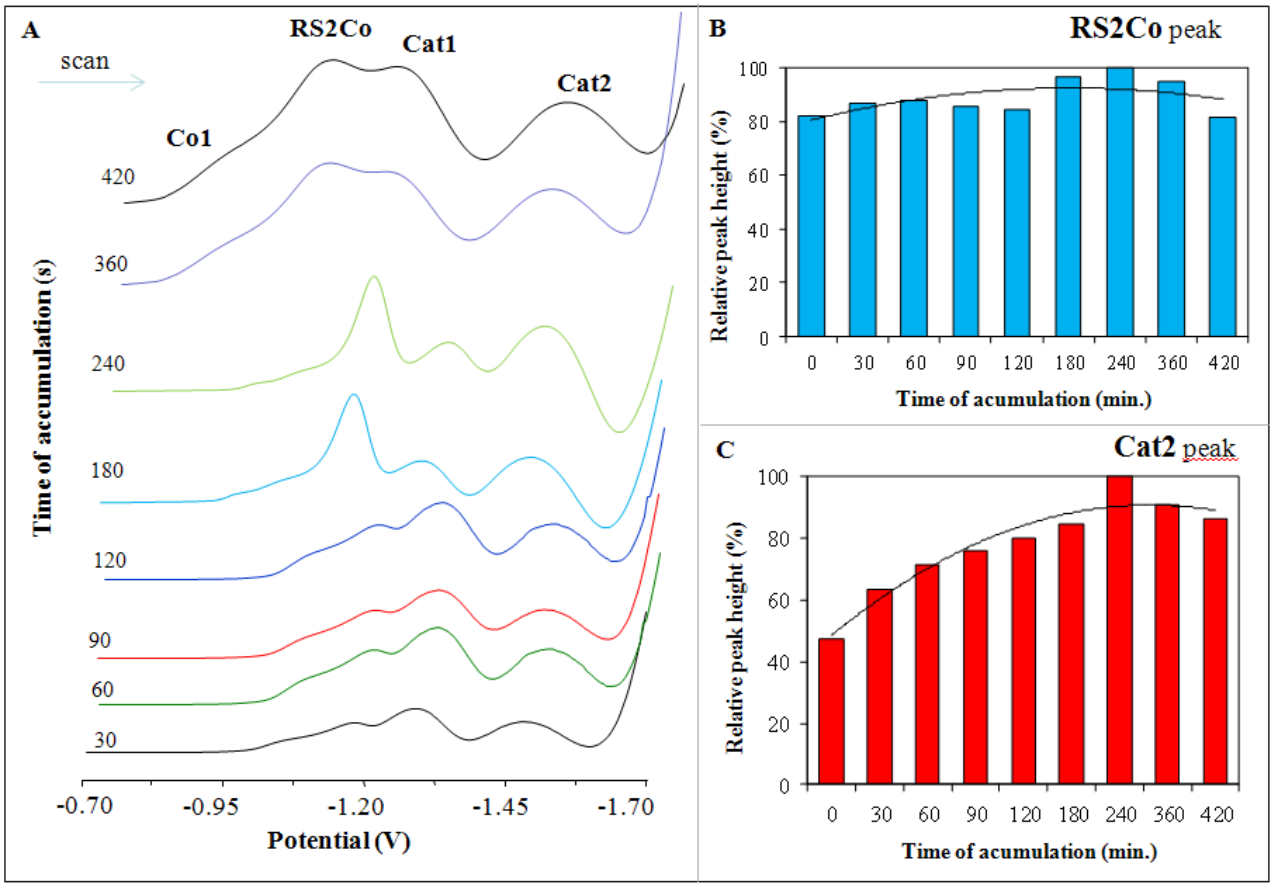

Figure 3. Electrochemical analysis of PSA in electrochemical cell. (**A**) Differential pulse voltammograms of PSA measured at 30, 60, 90, 120, 180, 240, 360 and 420 s accumulation time. Dependence of peaks heights of (**B**) RS2Co and (**C**) Cat 2 on accumulation time. DPV parameters were as follows: Initial potential -0.7 V; end potential -1.7 V; step potential 5 mV; modulation time 0.05 s, PSA 5 ng/ml, 1 mM Brdicka solution ($Co(NH_3)_6Cl_3$ + NH_3(aq) + NH_4Cl), pH = 9.6) as supporting electrolyte.

This peak is often proportional to concentration of a protein. There can be also detected peak called Co1 at higher concentrations of PSA. It follows from the results obtained that electrochemical processes occurring on the surface of HMDE induced by PSA are very complicated and will require further research associated with monitoring of structural changes in PSA during measurement. It can be assumed that the resulting observed phenomena are likely related to the relatively complex structure of PSA as a glycoprotein.

3.4 Effect of Brdicka solution on PSA detection

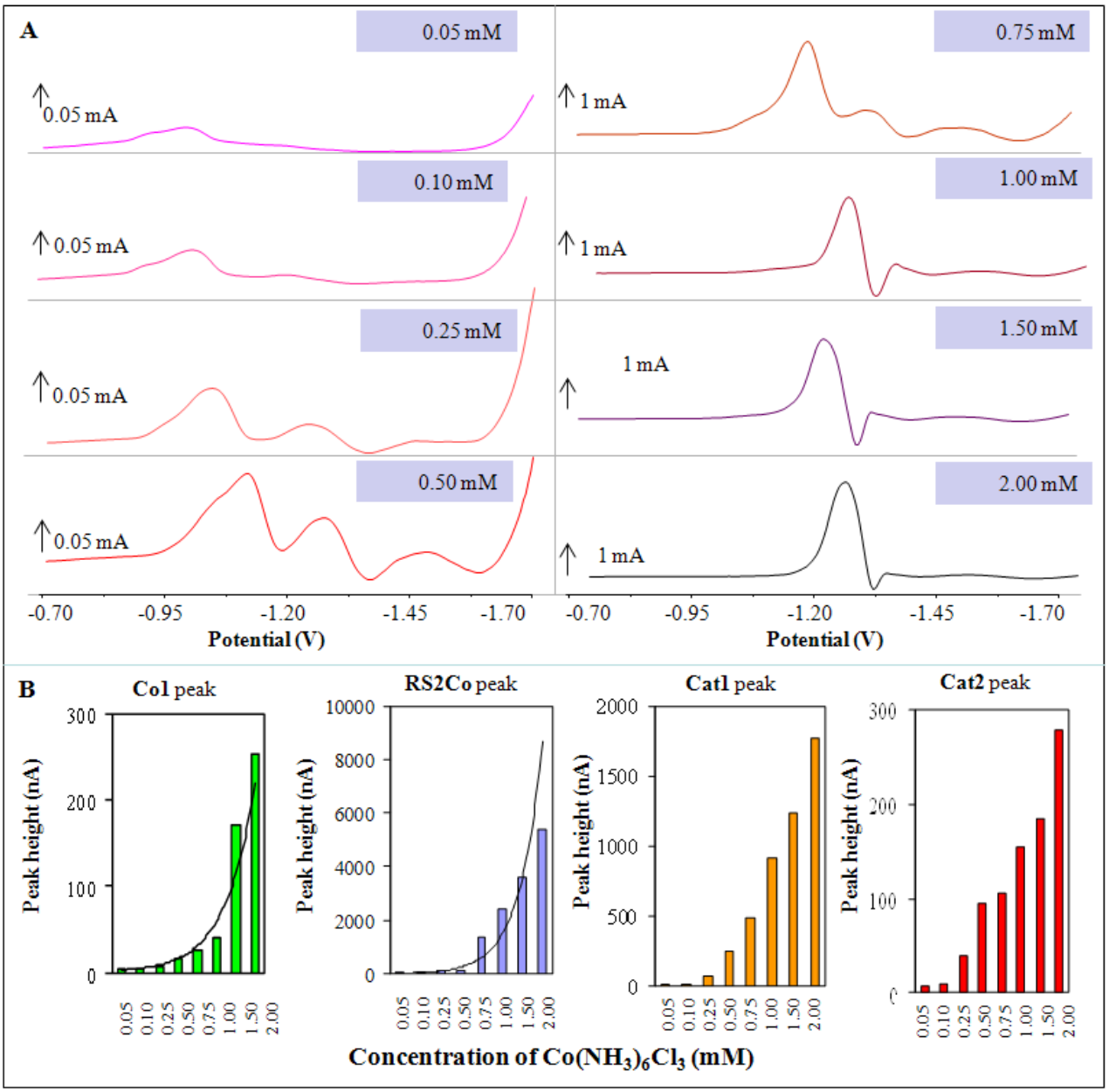

Figure 4. (**A**) Differential pulse voltammograms of PSA measured in the presence of Brdicka solution with various concentration of $Co(NH_3)_6Cl_3$ (0.05, 0.10, 0.25, 0.50, 0.75, 1.00, 1.50 and 2.00 mM). (**B**) Dependencies of peaks height (Co1, RS2Co, Cat1 and Cat2) on concentrations of cobalt(III) complex. For other experimental details see Fig. 3.

It is known that the concentration of components of a supporting electrolyte can markedly influence catalytic reaction [82,87,88]. Brdicka solution is based on a combination of ammonium ions, ammonium chloride and the cobalt(III) complex. Changes in the concentration of these components significantly affect the height of catalytic signals. Therefore, we were interested in the issue what changes in catalytic signals would be observed. We investigated the effect of various concentration of cobalt(III) complex (0.05, 0.10, 0.25, 0.50, 0.75, 1.00, 1.50 and 2.00 mM $Co(NH_3)_6Cl_3$) on catalytic signals of PSA (5 ng/ml). Typical DP voltammograms are shown in Fig. 4A. All detected signals enhanced with the increasing concentration of cobalt(III) complex, however, concentrations greater than 0.75 mM $Co(NH_3)_6Cl_3$ led to a dramatic increase in the peak associated with reduction of cobalt(II) ions in a solution of the supporting electrolyte. The changes in the heights of detected peaks as Co1, RS2Co, Cat1 and Cat2 with the increasing concentration of cobalt(III) complex are shown in Fig. 4B. Co1 peak dependence was well fitted with the exponential model ($y = 1.21^{e0.649x}$, R^2 = 0.9589), in which the highest peaks were detected in the presence of 1.50 and 2.00 mM $Co(NH_3)_6Cl_3$. Catalytic peak RS2Co (at -1.1 V) began to rise dramatically from the 0.75 mM concentration used. The obtained dependence is also of exponential character as $y = 1.21^{e0.649x}$, $R^2 = 0.9589$. Analytically useful signals Cat1 and Cat2 began to enhance more intensively in the presence of 0.25 mM. Dependence of Cat1 height on concentration of cobat(III) complex can be expressed by quadratic mathematical model $y = 42.272x^2 – 126.55x + 85.535$, $R^2 = 0.9981$. Cat2 showed linear increasing in the studied range of concentrations of $Co(NH_3)_6Cl_3$ as follows $y = 37.261x - 58.496$, $R^2 = 0.9447$. However, the trend better reflects the second-degree polynomial ($y = 3.6194x^2 + 4.6863x - 4.2055$, $R^2 = 0.9803$). Our results are in good agreement with Raspor et al. showing the similar dependencies of catalytic signals of MT on concentration of cobalt(III) complex [88].

3.5 Adsorptive transfer technique for PSA analysis

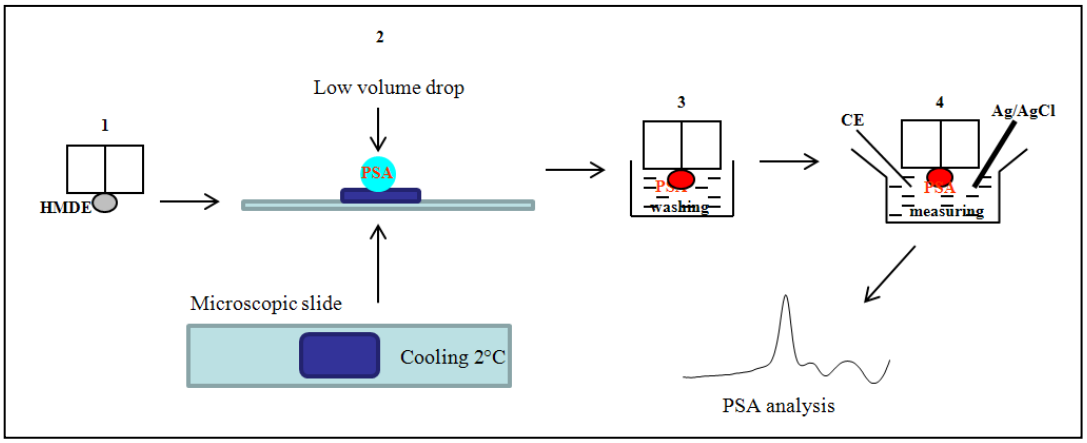

Figure 5. Scheme of improvement of the transfer to detect PSA in very low volumes of a sample. (**1**) Renewing of a surface of HMDE. (**2**) Microscopic slide, degreasing and seaming of small square from parafilm (10 × 10 cm, Sigma-Aldrich), transferring it to a beaker filled with distilled water and placed in the tempered water bath (temperature of 2 °C, at least 15 min., Julabo, Germany), drying it using cellulose and pipetting of a sample on it, (**3**) adsorbing of MT on the surface of HMDE, (**4**) transferring the electrode, and washing it with supporting electrolyte, transferring the electrode and measuring.

In our previously published study, we suggested a unique procedure for electrochemical analysis of very small volumes of a protein using cooling place for adsorption of a protein onto a surface of HMDE [46]. Despite the complexity of the electrochemical behaviour of the PSA, this was applied for PSA detection also. The principle of the approach is shown in Fig. 5. A small volume of sample is placed on parafilm placed on cooling slide, where evaporation is significantly limited and adsorption of PSA on the surface of HMDE is improved. After removal of non-specifically bound protein, the modified electrode is transferred into the working electrolyte (cooled to 4 °C), in which an electrochemical measurement is carried out (Fig. 5).

3.5.1 The influence of temperature on adsorption of PSA

The described technique was used for detection of PSA. We tested two temperatures for adsorption of the protein of interest as 4 °C and 20 °C at time of accumulation of 60 s. Primarily, we tested 20 °C and 5, 6, 7, 8, 9 and 10 µl volumes of PSA (5 ng/ml). The measured AdTS DP voltammograms are shown in Fig. 6A. Well developed signals of PSA were detected using 10 µl. The increasing volume of PSA caused disappearing of Cat1 signal and deformation of Cat2 signal and voltammograms obtained by analysis of lower volumes than 5 µl was poor and standard catalytic signals disappeared.

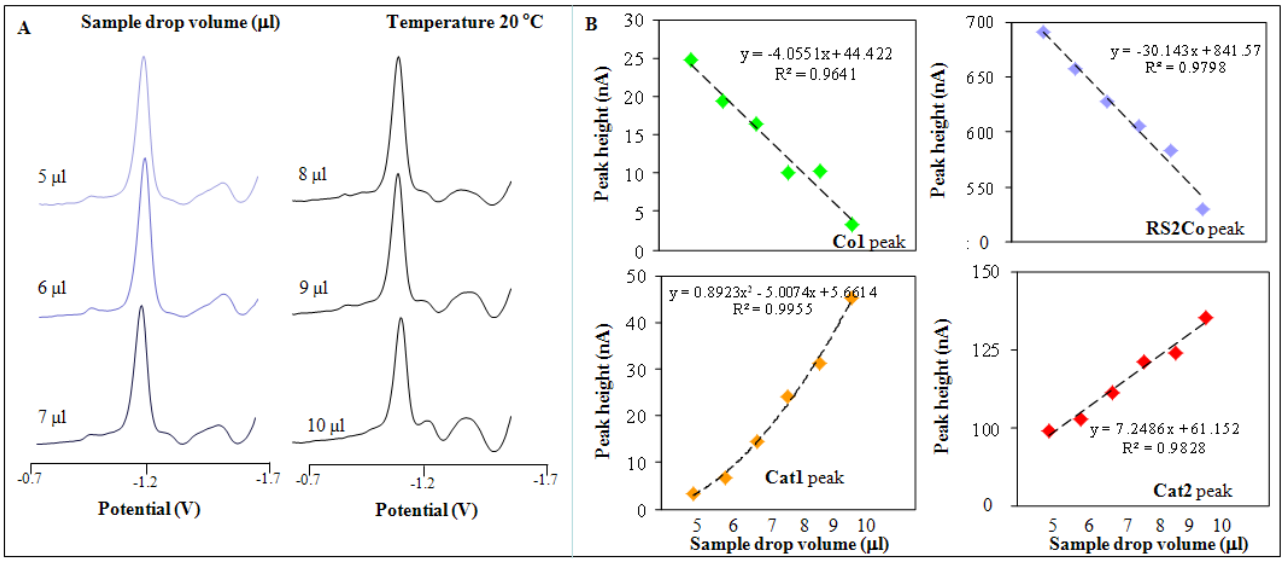

Figure 6. (**A**) AdTS differential pulse voltammograms of 5, 6, 7, 8, 9 and 10 µl PSA (5 ng/ml) measured in the presence of Brdicka solution (1 mM) and at 20 °C temperature of adsorption. (**B**) Dependencies of Co1, RS2Co, Cat1 and Cat2 peaks heights on volume of PSA. Time of accumulation 60 s. For other experimental details see Fig. 3.

The effect of various volumes on the height of detected signals is shown in Fig. 6B. Co1 and RS2Co decreased and Cat1 and Cat2 peaks increased with the increasing concentration and volume of PSA. Cat1 dependence can be well characterized by polynomial curve as $y = 0.8923x^2 - 5.0074x +$

5.6614, R^2 = 0.9955. Cat2 was proportional to volume of PSA, which can be expressed by the following equation y = 7.2486x + 61.152, R^2 = 0.9828. The results obtained confirm that PSA is well adsorbable on the surface of HMDE and Brdicka reaction can be used for its analysis.In the case of cooling of parafilm to 4°C the adsorption of PSA on the surface of HMDE was significantly improved and, thus, sensitivity can be markedly enhanced to reduce the amount of the sample needed for analysis. Typical AdTS DP voltammograms of 0.5, 1.0, 1.5, 2.5, 3.0 and 5.0 µl PSA (5 ng/ml) measured using HMDE with a drop area of 250 μm^2 at the time of accumulation 240 s are shown in Fig. 7A. Well reproducible voltammetric curves were detected during analysis of 2.5 µl of PSA (relative standard deviation was 7.2 %, n = 10). However, 0.5 µl sample of PSA can be well and reproducibly absorbed onto the surface of the HMDE and relative standard deviation did not exceed 20 %. The obtained dependencies of the catalytic peak heights on volume of analysed sample are shown in Fig. 7B and have a similar course as the adsorption at 20 °C. A significant difference is observed in the case of Cat2 peak, which dependence was strictly linear (y = 31.963x - 21.949, R^2 = 0.9930). Detection limit of PSA expressed as 3 S/N was estimated down to 2.5 pg of PSA in 0.5 µl.

3.5.2 The influence of time of adsorption on detection of PSA

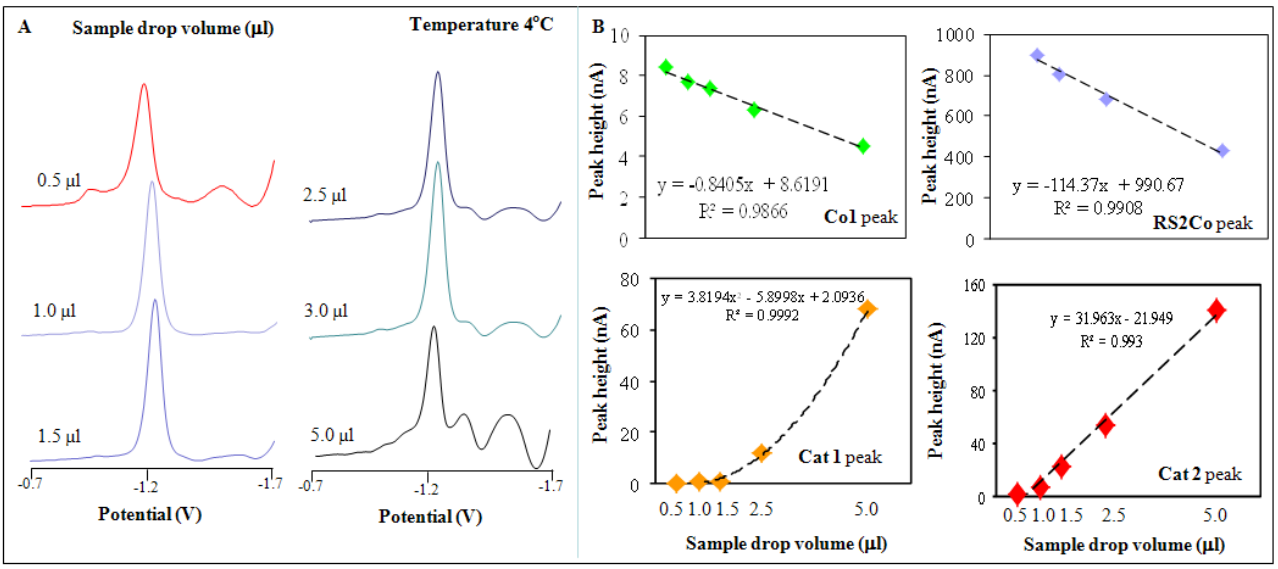

Figure 7. (**A**) AdTS differential pulse voltammograms of 0.5, 1.0, 1.5, 2.5, 3.0 and 5.0 µl PSA (5 ng/ml) measured in the presence of Brdicka solution (1 mM) and at 4 °C temperature of adsorption. (**B**) Dependencies of Co1, RS2Co, Cat1 and Cat2 peaks heights on volume of PSA. Time of accumulation 60 s. For other experimental details see Fig. 3.

Further, we aimed our attention on the studying of the effect of various times of adsorption on peaks of PSA. The temperature of adsorption was set at 4 °C based on the above mentioned results. The effect of various times of adsorption on the heights of catalytic signals of PSA (5 ng/ml) is shown in Fig. 8. The highest peaks were detected at 240 s long adsorption time except Co1, which did not change with the increasing time of adsorption. Longer accumulation time caused slight decrease in the

signals, which can be related to formation of some polylayers on the surface of HMDE. Both Cat1 and Cat2 peaks enhanced markedly after 240 s long adsorption compared to 60 s long adsorption. This was used to determine calibration curve with the following equation y = 36.865x - 11.949, R^2 = 0.9911 within the concentration interval from 1 to 1,500 pg/ml. Detection limit of PSA expressed as 3 S/N was estimated down to 1 fg of PSA in 0.5 µl.

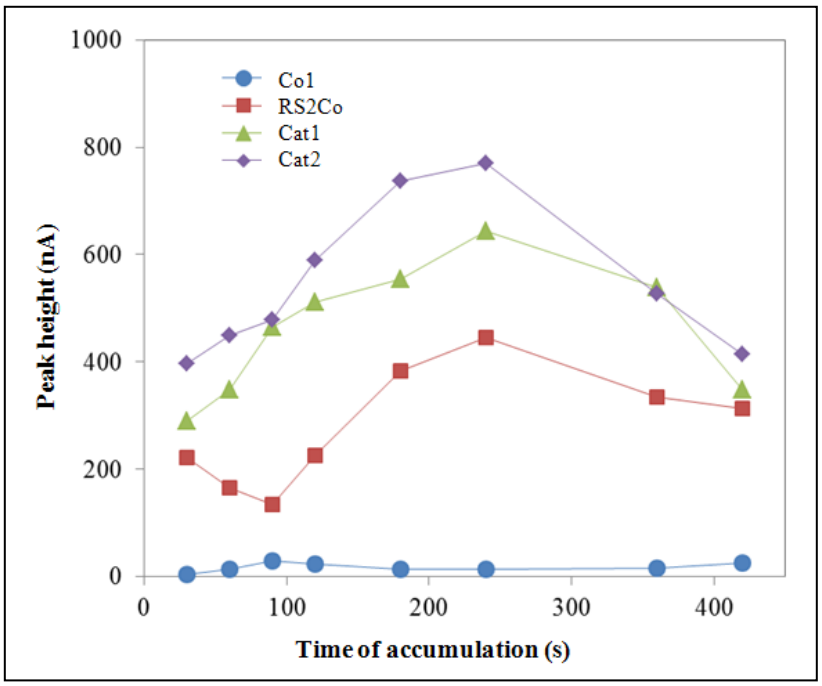

Figure 8. Dependence of Co1, RS2Co, Cat1 and Cat2 heights (on accumulation time. Temperature of adsorption 4 °C. For other experimental details see Fig. 3.

4. CONCLUSIONS

PSA still not sufficiently investigated from the point of view of its electrochemical properties and the results obtained in this study provides initial information. In addition, label-free detection techniques for protein analysis and/or microarrays are required because of their simple interpretation, less demanding sample preparation and thus also reduce the time and material consumption [89]. An interesting challenge for further detailed study of PSA is its interaction with zinc ions and changing its protease activity [90].

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