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Abstract: An electrochemical method for the determination of the catalytic activity of L-asparaginase (ASNase) from Erwinia carotovora was proposed. Our approach is based on the electrooxidation of amino acids from L-asparaginase polypeptide backbones. The electrochemical behavior of ASNase on electrodes obtained by screen-printing modified with single-wall carbon nanotubes (SPE/SWCNTs) as sensing elements demonstrated a broad oxidation peak at 0.5–0.6 V centered at 0.531 ± 0.010 V. We have shown that in the presence of the substrate L-asparagine, the oxidation current of the enzyme was reduced in a concentration-dependent manner. The specificity of electrochemical analysis was confirmed in experiments with glycine, an amino acid with no substrate activity on ASNase and does not reduce the oxidation peak of L-asparaginase. The addition of glycine did not significantly influence the amplitude of the oxidation current. The innovative aspects of the proposed electrochemical sensor are the direct monitoring of ASNase catalytic activity and a reagentless approach, which does not require additional reagents or labels.

Keywords: Erwinia carotovora L-asparaginase; electrochemistry; electro oxidation; screen-printed electrode; carbon nanotubes; protein electrochemistry; L-asparagine

1. Introduction

L-asparaginase (ASNase, L-asparagine amidohydrolase (EC 3.5.1.1)) has been used in medicine as a pharmacological antitumor agent for acute lymphoblastic leukemia [1,2], non-Hodgkin’s lymphoma and other malignant tumors, against pathogenic bacterial infection [3], and in the food industry for preventing acrylamide formation in processed foods with high starch content [1,4,5]. ASNase is an amidohydrolase that catalyzes the conversion of L-asparagine (L-Asn) into L-aspartic acid and ammonia. Tumor cells lack L-asparaginase synthetase and cannot synthesize L-Asp autonomously [6]. The deficiency of this amino acid can lead to abnormal protein synthesis in cancer cells and ultimately cause cell death [6]. Human cells cannot synthesize ASNase, and native L-ASNase from Escherichia coli (EcA) or Dickeya dadantii (formerly known as Erwinia chrysanthemi) (ErA) along with the pegylated form of E. coli asparaginase have been successfully used for the treatment of patients with acute lymphoblastic leukemia. The pharmacological dose of ASNase must be individually determined according to the patient’s clinical response and tolerance to ensure the maximum therapeutic effect and minimize side effects [6,7].

Estimation of ASNase catalytic activity is based on different approaches, such as the determination of L-Asn content or ammonia concentration measured by high-performance liquid chromatography (HPLC), direct amino acid quantification by circular dichroism...
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(2022), electrophoresis assays, and determination by a colorimetric assay from complexation with hydroxylamine [1]. The simultaneous production of L-aspartic acid (L-Asp) and NH4+ cations can also be used to quantify ASNase activity by conductometry, where the increase in conductivity corresponds to the rate of product formation [8,9]. The most commonly used Nessler reaction (2) with potassium tetraiodine mercurate (II) K2HgI4 requires using highly toxic reagents with poor stability. In addition, the Nessler reaction has low reproducibility and complicated operations and is not suitable for detecting clinical samples [6]. From these viewpoints, sensitive, reliable, and robust methods of determination and catalytic activity monitoring of L-asparaginase are in great demand.

Physical, physico-chemical, and nanotechnological approaches have also contributed to the development of detection methods for the estimation of enzyme catalytic activity. An approach to measuring the activity of monomers or oligomers of the heme-containing enzyme CYP102A1 by atomic force microscopy (AFM) has been developed. Using AFM, it was found that the amplitude of fluctuations of the height of single CYP102A1 molecules performing the catalytic cycle is twice as great as the amplitude of fluctuations of the height of the same enzymes in the inactive state [10]. A new powerful and versatile approach for measuring enzymatic activities based on the detection of biochemical events using nanopores has been employed [11]. However, this sensitive and elegant approach for monitoring biocatalysis requires additional equipment for the registration of low currents (in the 10^-12 A or 10^-15 A range) and suitable biological or inorganic nanopores.

Electrochemistry, and especially bioelectrochemistry, has great potential in the field of enzymology and determination of such events as substrate binding, the oxidation state of the catalytic center, and structural fluctuations [12,13].

Enzyme electrodes are analytical devices based on the combination of the high specificity of biocatalytic reactions with the electrochemical transduction of the recognition event [14–16]. Analysis of enzyme electrode kinetics is important for designing a sensor or for optimization of parameters for enzyme-substrate interactions [17,18].

Based on the analysis of the electrochemical parameters of the bioelectrode, such as the dependence of the catalytic current generated by the enzyme during concentration-dependent substrate conversion, quantitative calculations of the Michaelis constant Km, substrate constant Ks, maximum reaction rate Vmax, catalytic rate constants and catalytic efficiency can be calculated [17].

The electrochemical activity of protein molecules depends on two main structural characteristics, redox-active cofactors (heme or Flavin, for example) and electrochemically active amino acid residues [18–25]. Tyrosine, tryptophan, histidine, cysteine, cystine, and methionine yielded oxidation peaks at solid electrodes [19–23]. Amino acid oxidation is an irreversible electrochemical process. The changes in amino acid position and complex formation with metal ions or ligands influence the oxidation signal intensities. The electrochemical activity of amino acids is a valuable tool for the analysis and effective registration of protein post-translational modifications or “modificomics,” protein-function analysis, and detection of conformational changes [24–27]. Electrochemical profiling of acetylcholinesterase wild-type AChE and mutant proteins was performed [28]. Amino acid substitutions introduced through site-directed mutagenesis of AChE were detected using square-wave voltammetry on a screen-printed carbon electrode. The authors underlined that complex conformational changes of the polypeptide chain have a more dominant influence on the oxidation profile of the mutants than the individual amino acid substitutions [28].

As shown earlier, Thr12, Tyr25, Thr89, Asp90, and Lys162 of ASNase participate in catalysis and substrate binding [29–38]. These amino acids are conserved for all bacterial asparaginases [29,34–42]. The mechanism of catalysis involves two triads, Thr12-Lys162-Asp90, which resembles the classical serine proteinase mechanism and participates in catalysis, and Thr12-Tyr25-Glu283, which participates in substrate binding and releases products of catalytic reactions [35–37].

In our investigation, the ability and susceptibility of the protein to conformational changes registered by electrochemical oxidation were used to estimate the catalytic activity
of ASNase. We assumed that in the course of catalytic conversion of L-Asn as a substrate, structural fluctuations of ASNase occurred, which led to the rearrangement of the protein chain and changes in the intensities of electroactive amino acids as specific electrochemical labels [28]. These properties of a protein can be registered with voltammetric techniques such as differential pulse voltammetry.

2. Materials and Methods

2.1. Reagents and Materials

L-Asn and glycine (Gly) were purchased from Sigma–Aldrich (St. Louis, MO, USA). A water dispersion of 0.4% single-walled carbon nanotubes (SWCNTs, surface area 1000 m²/g) TUBALL™ BATT H₂O stabilized by carboxymethylcellulose obtained from OCSIAL Ltd. (Luxembourg) https://tuball.com/ru/additives) (accessed on 16 June 2022) was used. Asparaginase from Erwinia carotovora was prepared as described previously [38].

Screen-printed electrodes (SPE) with graphite working (geometric area 0.0314 cm²), auxiliary electrodes, and silver/silver chloride reference electrodes (Ag/AgCl) were obtained from ColorElectronics, Moscow, Russia (http://www.colorel.ru) (accessed on 16 June 2022).

2.2. Equipment

Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) measurements were performed using an Autolab PGSTAT12 potentiostat/galvanostat (Metrohm Autolab, Utrecht, The Netherlands) equipped with GPES software (version 4.9.7). All electrochemical experiments were carried out at room temperature in 100 mM potassium phosphate with 50 mM NaCl at pH 7.4. CV experiments were carried out in a 1 mL electrochemical cell by potential sweeping from an initial potential of −300 mV to an end-point potential of +800 mV at different scan rates in a range of 10–100 mV/s. DPV experiments were carried out in a 60 µL drop applied onto the electrode to cover all three electrodes.

Electrochemical studies of L-asparaginase were performed in 0.1 M potassium-phosphate buffer containing 0.05 M NaCl, pH 7.4. For the preparation of the modified electrodes, 2 µL of SWCNT in carboxymethylcellulose diluted with water as 1:2 (OCSIAL, Luxembourg, https://tuball.com/ru/additives) (accessed on 16 June 2022) was dropped onto the working area of the SPE and incubated for 15 min. All potentials were referenced to the Ag/AgCl electrode. All experiments were performed in triplicate. The data are presented as average values ± standard deviations.

For further incorporation of the analyte, a 60-µL aliquot (ASNase, 3 µg/µL of electrolyte buffer) or ASNase with an appropriate concentration of amino acids L-Asn or Gly was dispensed onto the electrode surface. All electrochemical experiments were carried out at room temperature (23 ± 3 °C). A horizontal regimen of measurement was used for all experiments. The DPV method was used with the following parameters: pulse amplitude, 25 mV; potential step, 1 mV; pulse duration, 50 ms. Binding of amino acids with ASNase was performed for 5 min at room temperature.

Modified SPE/SWCNTs have been utilized for a single measurement to avoid surface fouling, cross-contamination, and blockage of the electrode surface by protein oxidation products. Each sample was tested with three independent measurements. The relative standard deviation in all cases did not exceed 10%. In the figures, data points represent the mean values of the detected peak currents (Ip) or potential maxima (Emax), with the error bars showing the confidential intervals. Each voltammogram was processed with Savitzky–Golay level 3 smoothing and baseline corrected to a peak width of 0.003 V using the GPES moving average baseline correction tool. The oxidation peak current and peak potential values were then recorded for each measurement.

SEM micrographs of carbon nanotubes were obtained using a Hitachi S-5500 scanning electron microscope (Hitachi, Tokyo, Japan) operating at 25 kV.
3. Results and Discussion

Disposable screen-printed electrodes (SPEs) are widely used in electrochemical sensing of heme-proteins, drugs, DNA, drug-protein, and drug-DNA interactions [14,39–41]. They possess commercial availability, low cost, and the ability to modify the working surface with a broad choice of chemical materials with different structures. Carbon nanomaterials significantly improve the sensitivity of electrodes [34]. We used a drop-casting method to modify SPEs with a dispersion of SWCNTs in carboxymethylcellulose (SPE/SWCNT). Two-microliter drops of dispersion were applied to the working area of the SPE. As shown in Figure 1A,B, the images for SWCNTs demonstrated carbon nanomaterials as SWCNTs with an average diameter of 10 nm.

![Figure 1A](image1.png) ![Figure 1B](image2.png)

**Figure 1.** SEM images of the water dispersion of single-wall carbon nanotubes (SWCNTs) stabilized by carboxymethylcellulose at higher (A) and lower (B) magnitudes.

Cyclic voltammetry (CV) measurements were used to assess the electroactive surface area of the modified SPE/SWCNTs. Figure 2A demonstrates the CV profiles for the ferri/ferro cyanide $[\text{Fe(CN)}_6]^{3-/4-}$ redox probe with a couple of well-defined redox peaks for SPE/SWCNT. The linear dependence of the peak current versus the square root of the scan rate was found and confirmed as a diffusion-controlled process (Figure 2B) [42]. The anodic/cathodic peak currents for the SPE/SWCNT have much higher intensities than bare SPE with a lower $\Delta E$ peak-to-peak separation (Figure 2C). This indicates that a more reversible redox performance of $[\text{Fe(CN)}_6]^{3-/4-}$ occurs when CNTs are used. SPE/SWCNTs possess a larger specific surface area according to the Randles–Sevcik equation [42]. The calculated values of the specific surface area corresponded to 0.0024 cm$^2$ and 0.1258 cm$^2$ for SPE and SPE/SWCNT, respectively. These results confirmed the good conductivity and electron transfer properties of carbon nanomaterials such as carbon nanotubes [14,39–42].
Figure 2. (A) Typical CV curves for SPE/SWCNT. The measurements were carried out in 5 mM K$_3$Fe(CN)$_6$ at ambient temperature in a potential range from $-400$ mV to $+800$ mV (vs. Ag/AgCl) at scan rates in the range of 10–100 mV/s. (B) The dependence of peak current $I_p$ on the square root of the scan rate in the range of 10–100 mV/s. (C) The typical CV curves for SPE (--) and SPE/SWCNT (--) in 5 mM K$_3$Fe(CN)$_6$ at 50 mV/s.
SPEs modified with SWCNTs (SPE/SWCNTs) were used for the electrochemical registration of ASNase. ASNase was deposited onto the modified active area of the working electrode. This step was accomplished by the direct adsorption method. As we have shown earlier, modification of the SPE with CNTs significantly enhances the ability of the electrode to detect amino acid and protein electrochemical oxidation [27]. To obtain the electrochemical response of ASNase on SPE/SWCNTs, we used a differential pulse voltammetry technique (DPV), which permits the registration of enzymes with good sensitivity. (Figure 3A). Electrochemical profiling of ASNase on SPE/SWCNT revealed a broad oxidation peak in the 0.5–0.6 V with a maximum amplitude of 0.593 ± 0.007 V. The oxidation peak was detected using DPV and corresponded to the irreversible oxidation of electroactive tyrosine (Tyr), tryptophan (Trp) and cysteine (Cys) residues [21–25,29,30,42]. No redox signals were observed for the SPE/SWCNTs in the absence of protein, confirming that the oxidation peak is attributed to ASNase. SPE/SWCNTs with two concentrations of ASNase (3 µg/µL and 5 µg/µL) also confirmed the nature of the electrochemical signal of the enzyme (Figure 3A). Sensitivity towards asparaginase was determined as 0.80 ± 0.07 µA/µg/µL of protein.

Figure 3. (A). DPV electrochemical oxidation signals of SPE/SWCNT/ASNase, 3 µg/µL (-), and 5 µg/µL (-), SPE/SWCNT (—). (B). DPVs of the first (-) and the second (—) scan of SPE/SWCNT/ASNase (5 µg/µL). (C) DPVs of SPE/SWCNT/ASNase, 3 µg/µL (-), 33 mM L-Asn (-), and 33 mM Gly (-) on SPE/SWCNT, SPE/SWCNT (—). Supporting electrolyte: 0.1 M potassium-phosphate buffer containing 0.05 M NaCl, pH 7.4.

The second scan of DPV for ASNase on SPE/SWCNT revealed a lower peak current intensity, thus confirming the irreversible nature of amino acid electrooxidation (Figure 3B).
Structural rearrangements of proteins and especially hemoproteins during substrate binding can be registered using spectroscopy [43–45]. Upon substrate or inhibitor interaction with the iron porphyrin of cytochrome P450, spectral changes in the Soret band were observed [44]. The electrochemical response of heme proteins upon substrate binding may be registered as the appearance of catalytic current and shift of potential [17,18,46]. For enzymes without prosthetic groups, conformational changes in the polypeptide backbone registered using electrochemical oxidation of amino acids may be used as an effective tool for the investigation of enzyme-substrate interactions. The electrochemical signature of the enzyme based on the electrochemical oxidation of electroactive amino acids permits its use as an indicator for the assessment of the catalytic activity of ASNase in the presence of amino acids L-Asn and Gly. These amino acids have no significant electrooxidation properties on SPE/SWCNTs in the studied range of potentials (Figure 3C).

Dynamic conformational changes of enzymes during enzyme catalysis and interaction with the substrate are crucial to the catalytic activities and for the registration of these changes using the electrochemical analysis of such changes. Substrate binding was performed for 5 min at room temperature. Furthermore, DPV was employed to follow the direct electrooxidation of ASNase and its subsequent interaction with amino acids. The interplay of ASNase with L-Asn shows a significant decrease in the oxidation current (Figure 4A,B). A slight shift in reduction potential was registered for the interaction of ASNase with the substrate L-asparagine (Table 1). Significant differences in the registered oxidation behavior of ASNase were observed in the presence of L-asparagine, L-glutamine, and glycine, which has no substrate properties (Figure 4). Glycine at the same concentration did not produce significant conformational changes in the polypeptide chain, did not change the oxidation potential, and did not significantly influence the oxidation amino acid current, as confirmed by DPV (Figure 4B, Table 1). L-glutamine decreased the oxidation current of ASNase, by 43 percent in comparison with 23 percent in the presence of L-Asn (Figure 4B and Table 1), confirming the preference of the enzyme for L-Asn. The electrochemical approach permits to discriminate catalytic activity of enzyme ASNase towards amino acids with different roles in catalysis.

A concentration-dependent decrease in the oxidation current of ASNase was used to assess the Michaelis constant for substrate L-Asn. Electrochemical data were transformed into kinetic data using a nonlinear regression method using the OriginPro (version 8.5, Northampton, MA, USA) software package ($R^2 = 0.968$). Curves were fitted to obtain the $K_m$ value [47,48].

$$I_{\text{cat}} = \frac{I_{\text{cat max}} [S]}{K_m + [S]}$$

Based on such dependencies, the electrochemical Michaelis constant for L-Asn was calculated and corresponded to $600 \pm 70 \mu M$ (Figure 5). This parameter was calculated as $98 \mu M$ using the Nessler reagent [49]. The most commonly used Nessler reaction requires the use of highly toxic reagents with poor stability, and the Nessler reaction has low reproducibility and complicated operations and is not suitable for the detection of clinical samples [6]. The electrochemical approach depends on the type of electrode, electrode modifier, and accessibility of substrate to the active center of the enzyme. For these reasons, the difference between the $K_m$ value obtained using the Nessler reaction and the electrochemical approach may be registered. The fluorescence analysis method requires the synthesis of a fluorogenic probe for the assessment of the catalytic activity of the enzyme. From this viewpoint, the electrochemical approach is the most robust and effective.

We assumed that amino acids with the ability for electrochemical oxidation, such as Tyr, Trp, His, Cys, Cys-Cys, and Met [19–27], are responsible for the response to substrate interactions. Tyr29 is a crucial amino acid in the active site of bacterial L-asparaginases and Tyr29 is the most important candidate for the main role in electrochemical registration of enzyme-substrate interactions in ASNase from *Erwinia carotovora* [31,47]. Tyr29 of L-asparaginase from *Erwinia carotovora* is involved in substrate binding and release of reaction product [31].
Figure 4. (A) DPV electrochemical oxidation signals of SPE/SWCNT/ASNase (-), SPE/SWCNT/ASNase after interaction with 33 mM Asn (-), SPE/SWCNT (—). (B) Histograms corresponding to the average ASNase signals on SPE/SWCNT and ASNase on SPE/SWCNT after interaction with 33 mM L-Asn, after interaction with 33 mM Gly, after interaction with 33 mM L-Gln. Supporting electrolyte: 0.1 M potassium-phosphate buffer containing 0.05 M NaCl, pH 7.4.

Table 1. Data from the differential pulse voltammetric (DPV) measurements of ASNase and ASNase after incubation with L-Asn, L-Gln, or Gly at SPE/SWCNTs (n = 3).

| SPE/SWCNT          | Peak Potential, E (V) | Peak Current, Ip (µA) | Peak Width (1/2) | Title 1 | Title 2 | Title 3 | Title 4 |
|--------------------|-----------------------|-----------------------|------------------|---------|---------|---------|---------|
| ASNase             | 0.593 ± 0.007         | 1.72 ± 0.22           | 0.166            | data    | data    | data    | data    |
| ASNase + 33 mM L-Asn | 0.588 ± 0.002       | 0.39 ± 0.18           | 0.151            | entry 1 | data    | data    | data    |
| ASNase + 33 mM Gly | 0.593 ± 0.007         | 1.80 ± 0.10           | 0.166            | data    | data    | data    | data    |
| ASNase +33 mM L-Gln | 0.596 ± 0.006       | 0.74 ± 0.20           | 0.166            | entry 4 | data    | data    | data    |

* For substrate binding, ASNase was incubated with L-asparagine, L-glutamine, or glycine for 5 min.
Figure 5. The dependence of the difference in the asparaginase oxidation current $\Delta I = I_{\text{enz}} - I_{(\text{enz} + \text{Asn})}$ vs. L-Asn concentration as an electrochemical equivalent of the Michaelis–Menten plot. The values are the means from at least 3 experiments $\pm$ S.D.

4. Conclusions

The catalytic activity of ASNase from *Erwinia carotovora* was registered electrochemically using the DPV technique. The electrooxidation of amino acids from L-asparaginase backbones was used as a measuring tool. ASNase was immobilized on SPE modified by carbon nanotubes. The substrate L-asparagine reduced the oxidation current of the enzyme in a concentration-dependent manner. The electrochemical L-asparagine Michaelis constant corresponded to 600 $\pm$ 70 µM and was the same order of magnitude as the biochemical value, calculated with analysis of product formation measured with Nessler reagent. The addition of glycine did not significantly influence the amplitude of the oxidation current. The proposed electrochemical method permits the registration of direct ASNase electrochemical oxidation for monitoring substrate binding and does not require specific labels of protein or substrate. However, the electrochemical approach depends on the type of electrode, electrode modifier, and accessibility of substrate to the active center of the enzyme. The future perspective of this method is the construction of new nanocomposite materials for the modification of working electrodes with enhanced sensitivity to the electrochemical oxidation of amino acids, investigation of mutant forms of asparaginase from different sources, a range of amino acids, and inhibitors of this enzyme.

Author Contributions: Conceptualization, V.S. and D.Z.; methodology, V.S. and T.B. designed the experiments and analyzed the obtained data. Formal analysis and investigation: T.B., V.P. and V.S. performed the electrochemical experiments, S.K. performed the SEM experiments. Writing—original draft preparation: V.S. prepared an original draft. Writing—review and editing, D.Z. carried out a review and editing. M.P. and S.A. cloned and purified L-asparaginase. Supervision, V.S. and D.Z. All authors have read and agreed to the published version of the manuscript.

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