Electroenzymatic model system for the determination of catalytic activity of Erwinia carotovora L-asparaginase

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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 that has 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.

Introduction

L-asparaginase (ASNase) has been used in medicine as a pharmacological antitumor agent for acute lymphoblastic leukemia (Margi et al. 2018, Hill et al. 1967), non-Hodgkin's lymphoma and other malignant tumors, against pathogenic bacterial infection (Vimal & Kumar 2017), and in the food industry for preventing acrylamide formation in processed foods with high starch content (Margi et al. 2018, Aiswarya & Baskar et al. 2018, Bleyer et al 2002). ASNase is an amidohydrolase that catalyzes the conversion of L-asparagine (L-Asn) into L-aspartic acid and ammonia. Tumor cells lack L-asparagine synthetase and cannot synthesize L-Asn autonomously (Endicott et al. 2021, Wang et al. 2021). Deficiency of this amino acid can lead to abnormal protein synthesis in cancer cells and ultimately cause cell death (Wang et al. 2021). Human cells cannot synthetize 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 needs to be individually determined according to the patient's clinical response and tolerance to ensure the maximum therapeutic effect and minimize side effects (Wang et al. 2021, Karpel-Massler et al. 2016).

Estimation of ASNase catalytic activity is based on different approaches, such as determination of L-Asn content or ammonia concentration measured by high-performance liquid chromatography (HPLC), direct amino acid quantification by circular dichroism (CD) (Kudryashova & Sukhoverkov 2016), electrophoresis assays, and determination by a colorimetric assay from complexation with hydroxylamine (Magri et al. 2018). The simultaneous production of L-aspartic acid (L-Asp) and NH$_4^+$ cations can also be used to quantify ASNase activity by conductometry, where the increase in conductivity corresponds to the rate of product formation (Kudryashova et al.2016, Drainas & Drainas 1985). The most commonly used Nessler reaction with potassium tetraiodine mercurate (II) K$_2$Hgl$_4$ requires the use of highly toxic reagents with poor stability. In addition, the Nessler reaction has low reproducibility and complicated operations
and is not suitable for the detection of clinical samples (Wang et al. 2021). 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. Thus, the detection of microwave radiation of cytochrome P450 102A1 (CYP102 A1) and house reddish peroxidase during the enzyme reactions were described as measuring tools for the assessment of catalytic activity (Ivanov et al. 2015, Ivanov et al. 2016). An approach to measure the activity of monomers or oligomers of the heme-containing enzyme CYP102A1 by atomic force microscopy (AFM) has been developed. It was found using AFM, 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 (Ivanov et al. 2011). A new powerful and versatile approach for measuring enzymatic activities based on the detection of biochemical events by means of nanopores has been employed (Sheng et al. 2020). 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, oxidation state of the catalytic center, and structural fluctuations (Wang et al. 2008, Davis et al. 2021).

Enzyme electrodes are analytical devices based on the combination of the high specificity of biocatalytic reactions with the electrochemical transduction of the recognition event (Ferrari et al. 2021, Ghosh et al. 2015, Ambaye et al. 2021). Analysis of enzyme electrode kinetics is important for designing a sensor or for optimization of parameters for enzyme-substrate interactions (Shumyantseva et al. 2018, Shumyantseva et al. 2020).

Based on the analysis of the electrochemical parameters of bioelectrodes, such as the dependence of the catalytic current generated by enzymes during concentration-dependent substrate conversion, quantitative calculations of the Michaelis constant $k_m$, substrate constant $K_s$, maximum reaction rate $V_{max}$, catalytic rate constants and catalytic efficiency can be calculated (Shumyantseva et al. 2018).

The electrochemical activity of protein molecules depends on two main structural characteristics, redox-active cofactors (heme or Flavin, as an example) and electrochemically active amino acid residues (Shumyantseva et al. 2020, Brabec et al. 1980, Malfoy et al. 1980, Reynaud et al. 1980, Shumyantseva et al. 2014, Reinders and Sickmann 2007). Tyrosine, tryptophan, histidine, cysteine, cystine and methionine yielded oxidation peaks at solid electrodes (Brabec et al. 1980, Malfoy & Reynaud 1980, Reynaud et al. 1980). 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 posttranslational modifications or “modifomics”, protein function analysis, and the detection of conformational changes (Shumyantseva et al. 2014, Reinders and Sickmann 2007, Wei et al 2012, Shumyantseva et al. 2018). Electrochemical profiling of acetylcholinesterase wild-type AChE and mutant proteins was performed (Somji et al. 2012). 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 (Somji et al. 2012).

It is well known that enzymes specifically interact with their substrates by the “lock and key” Koshland model and by the “induced fit” model. Such behavior of enzymes during catalysis can be registered by means of different techniques. In our investigation, the ability and sensitivity of amino acids of the polypeptide backbone to conformational changes 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 (Somji et al. 2012). These properties of protein can be registered with voltammetric techniques such as differential pulse voltammetry.

Materials And Methods

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. (https://ocsial.com) was used. Asparaginase from Erwinia carotovora was prepared as described previously (Papageorgiou et al.2008).

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, Russia (http://www.colorel.ru).

Equipment

Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) measurements were performed using an Autolab PGSTAT12 potentiostat/galvanostat (Metrohm Autolab, 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. Three-pronged electrodes obtained by screen printing (SPEs) with graphite working and auxiliary electrodes and silver/silver chloride (Ag/AgCl) reference electrodes were used. The diameter of the working electrode was 0.2 cm (geometric area 0.0314 cm$^2$).

For preparation of the modified electrodes, 2 µl of SWCNT in carboxymethylcellulose diluted with water as 1:2 (OCSiAl, https://tuball.com/ru/additives) was dropped onto the working area of the SPE and incubated for 15 min. Electrochemical measurements were performed at room temperature in 0.1 M potassium phosphate buffer (pH 7.4) containing 0.05 M NaCl. All potentials refer 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. The enzymatic reaction of ASNase was performed for 5 min at 37°C.

Modified SPE/SWCNTs have been utilized for a single measurement to avoid surface fouling, cross-contamination and blockage of the electrode surface by oxidation products of protein. 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. Oxidation peak current and peak potential values were then recorded for each measurement. Electrochemical data were transformed to kinetic data by means of a nonlinear regression method using the OriginPro (version 8.5) software package. Curves were fitted to obtain the km value.

SEM micrographs of carbon nanotubes were obtained using a Hitachi S-5500 scanning electron microscope operating at 25 kV.

Results And Discussion

Disposable screen-printed electrodes (SPEs) are widely used in electrochemical sensing of hemeproteins, drugs, and DNA, drug-protein, and drug-DNA interactions (Ferrari et al. 2021, Sigolaeva et al. 2019, Manna et al. 2022, Carrara et al. 2014). 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 (Carrara et al. 2013). We used a drop-casting method for the modification of SPEs with a dispersion of SWCNTs in carboxymethylcellulose.
(SPE/SWCNT). Two ml drops of dispersion were applied to the working area of the SPE. As shown in Fig. 1, the images for SWCNTs demonstrated carbon nanomaterials as SWCNTs with an average diameter of 10 nm.

Cyclic voltammetry (CV) measurements were used to assess the electroactive surface area of the modified SPE/SWCNTs. Fig. 2A demonstrates the CV profiles for the ferri/ferro cyanide Fe(CN)$_6^{3-/4-}$ redox probe with a couple of well-defined redox peaks for SPE/SWCNT. The anodic/cathodic peak currents for the SPE/SWCNT have much higher intensities than bare SPE with a lower DE peak-to-peak separation (Fig. 2B). This indicates that a more reversible redox performance of [Fe(CN)$_6^{3-/4-}$ occurs when SCNTs are used. SPE/SWCNTs possess a larger specific surface area according to the Randles-Sevcik equation (Wang 2006) (Fig. 2C). 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 (Ferrari et al. 2021, Sigolaeva et al. 2019, Manna et al. 2022, Carrara et al. 2013).

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 SPE with CNTs significantly enhances the ability of the electrode to detect amino acids and proteins during electrochemical oxidation (Shumyantseva et al. 2018a). 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. (Fig. 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 (Y), tryptophan (W) and cysteine (C) residues (Brabec et al.1980, Malfoy et al.1980, Reynaud et al.1980, Shumyantseva et al. 2018a, Somji et al. 2012, Manna et al. 2022). 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 (Fig. 3A).

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 (Fig. 3B).

Structural rearrangements of proteins and especially hemoproteins during substrate binding can be registered by means of spectroscopy (Schenkman et al. 1967, Guengerich et al. 2021, Guengerich et al. 2020). Upon substrate or inhibitor interaction with the iron porphyrin of cytochrome P450, spectral changes in the Soret band were observed. The formation of the enzyme-substrate complex affects the spin equilibrium of cytochrome P450. Type I substrates induce a high-spin shift of ferric heme, and type II substrates convert heme iron into a low-spin form. These spectral features reflect conformational changes of porphyrin and spin shifts of heme iron (Guengerich et al. 2021). The electrochemical response of heme proteins upon substrate binding may be registered as the appearance
of catalytic current and shift of potential (Shumyantseva et al. 2020, Schneider and Clark 2013, Shumyantseva et al. 2018b). For enzymes without prosthetic groups, conformational changes in the polypeptide backbone registered by means of 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 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/SWCNT in the studied range of potentials (Fig. 3A, inset).

Dynamic conformational changes of enzymes during enzyme catalysis and interaction with substrate are crucial to the catalytic activities and for the registration of these changes by means of 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 (Fig. 4). A slight shift in reduction potential was registered for the interaction of ASNase with the substrate L-asparagine (Table 1). Glycine at the same concentration did not produce significant conformational changes in the polypeptide chain, did not change the oxidation potential of protein and did not significantly influence the oxidation amino acid current, as confirmed by DPV (Table 1).

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

<table>
<thead>
<tr>
<th>SPE/SPE/SWCNT</th>
<th>Peak Potential, E (V)</th>
<th>Peak Current, Ip (µA)</th>
<th>Peak Width (1/2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASNase</td>
<td>0.593±0.007</td>
<td>1.72±0.22</td>
<td>0.166</td>
</tr>
<tr>
<td>ASNase +33 mM L-Asn*</td>
<td>0.588±0.002</td>
<td>0.39±0.18</td>
<td>0151</td>
</tr>
<tr>
<td>ASNase +33 mM Gly*</td>
<td>0.593±0.007</td>
<td>1.80±0.10</td>
<td>0.166</td>
</tr>
</tbody>
</table>

*For substrate binding, ASNase was incubated with L-asparagine or glycine for 5 min.

A concentration-dependent decrease in the oxidation current of ASNase was used for the assessment of the Michaelis constant for substrate L-Asn. Based on such dependencies of the electrochemical Michaelis constant, km for L-Asn was calculated and corresponded to 400 µM (Fig. 5). This parameter was calculated as 98 µM using Nessler reagent (Krasotkina et al. 2004).

Significant differences in the registered oxidation behavior of ASNase were observed in the presence of the substrates L-asparagine and glycine, which have no substrate properties (Fig. 4). We assumed that amino acids with electrochemical oxidation abilities, such as tyrosine, tryptophan, histidine, cysteine, cystine and methionine (Brabec et al.1980, Malfoy et al.1980, Reynaud et al.1980, Shumyantseva et al. 2018a, Somji et al. 2012, Manna et al.2022), are responsible for the response to substrate interactions. Tyr25 is the most important candidate for the main role in electrochemical registration of enzyme-substrate interactions in ASNase from Erwinia carotovora.

**Conclusion**

The catalytic activity of ASNase from Erwinia carotovora was registered electrochemically by means of the DPV technique. The electrooxidation of amino acids from L-asparaginase backbones was used as a measuring tool. ASNase was immobilized on SPE and 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 400 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.

**Declarations**

**Acknowledgments**

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**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.V. and V.S. performed the electrochemical 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 A.S. cloned and purified L-asparaginase. Supervision: V.S. and D.Z. All authors have read and agreed to the published version of the manuscript.

**Conflict of interest** The authors declare no conflict of interest.

**Research involving human participants and animals** The research does not involve any human or animal studies.

**Informed consent** Not applicable.

**References**


Figures
Figure 1

SEM micrographs of the water dispersion of single-wall carbon nanotubes (SWCNTs) stabilized by carboxymethylcellulose
Figure 2

(A) Typical CV curves for SPE/SWCNT. The measurements were carried out in 5 mM \( \text{K}_3\text{Fe(CN)}_6 \) 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): Typical CV curves for SPE (−) and SPE/SWCNT (−) in 5 mM \( \text{K}_3\text{Fe(CN)}_6 \) at 50 mV/s.
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 second scans 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

Figure 4

(a)

(b)
DPV electrochemical oxidation signals of SPE/SWCNT/ASNase (-), SPE/SWCNT/ASNase after interaction with 33 mM Asn (-), SPE/SWCNT/ASNase after interaction with 33 mM Gly (-), SPE/SWCNT (--). Supporting electrolyte: 0.1 M potassium-phosphate buffer containing 0.05 M NaCl, pH 7.4

Figure 5

Michelis plot of the dependence of L-asparagine concentration vs. peak current of DI = (ASNase – ASNase+L-Asn). The dependence of the difference in the asparaginase oxidation current DI =I_{enz} –I_{enz+Asn} vs L-Asn concentration as an electrochemical equivalent of the Michaelis–Menten plot. The values are the means from at least 3 experiments ± S.D.