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Developing an interesting electrochemical biosensing system from an enzyme inhibition study: Binding, inhibition and determination of catalase by ascorbate

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Abstract

By this article, we are going to report results of one of works which has been performed on investigation of the binding and inhibition of catalase (CAT) by ascorbate (ASC). To achieve this goal, different electrochemical experiments have been performed and their data have been analyzed by conventional and chemometric methods. Conventional methods were including direct analysis of the electrochemical data by observation of them and using simple mathematical equations while chemometric analyses of the electrochemical data helped us to obtain more information which completed the previous information and gave us a new insight to the binding of the ASC with CAT. The next step of our study was devoted to the investigation of the binding of ASC with CAT by molecular docking methods which gave us new information about binding and inhibition of the CAT by ASC. All the steps gave specific information which not only confirmed each other but also gave new information which helped us to better understanding the mechanism of the binding and inhibition of the CAT by ASC. Finally, based on inhibition of the CAT by ASC, we have developed a novel impedimetric method for determination of the CAT.

Keywords: Catalase; Ascorbate; Inhibition.

1. Introduction

Catalase (CAT) is an enzyme which could be found in all living organisms exposed to oxygen which is able to decompose hydrogen peroxide to water and oxygen [1]. The CAT is very important because it is able to protect the cells from reactive oxygen species which are able to cause oxidative damage to the living cells. There is some evidence on inhibition of the CAT by ascorbate (ASC) [2,3]. ASC is a vital vitamin which could be found in a variety of foods, fruits and supplements. Therefore, it would be very interesting to investigate the inhibitory effects of the ASC on CAT.

Immobilization is a key parameter to optimize the operational performance of an enzyme [4,5]. There are different methods for immobilization of enzymes which are divided into three main categories including (i) binding to a prefabricated support (carrier), (ii) entrapment in organic or inorganic polymer matrices, and (iii) cross-linking of enzyme molecules [4,5]. Immobilization of enzymes allows us to re-use the enzyme for an extended period of time and improves many properties of enzymes such as increasing the structural rigidity and stabilization of multimeric enzymes which prevents dissociation-related inactivation.

Investigation of the binding of a small molecule with a biological macromolecule could be performed by using different instrumental techniques such as UV-Vis [6], FT-IR [7], capillary electrophoresis [8], HPLC [9], NMR [10] and electrochemistry [11-17]. Each one of these instrumental techniques is interesting and could be used to investigate binding of a small molecule with a biological macromolecule, but they could be more interesting when they are assisted by chemometric methods. Chemometrics is a new field in analytical chemistry which enables the analytical chemists to process their data to extract more information which could not be obtained by the use of conventional methods [18-24]. Among the available methods for investigation of the binding of a small molecule with a biological macromolecule, electrochemical methods are very interesting particularly when they are assisted by chemometric methods [11-17].

Therefore, in this work, we are going to investigate binding and inhibition of the CAT by ASC with the help of electrochemical techniques assisted by chemometric methods. The data will be analyzed by conventional and chemometric methods to obtain some information about the mentioned interactions. Chemometric hard- and

soft-modeling methods will help us to obtain additional information to confirm the results obtained by conventional methods and also to cast a deep look on binding and inhibition of the CAT by ASC. Then, molecular docking techniques will be performed to obtain theoretical information to expand our knowledge about inhibition of the CAT by ASC and all the information will be discussed and concluded. Finally, according to the inhibition of the CAT by ASC, a novel impedimetric method will be developed for determination of the CAT. The steps described above have been schematically summarized in Scheme 1.

Scheme 1

2. Experimental and theoretical details

2.1. Chemicals and solutions

CAT, ASC, graphene (Gr), 1-ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide (IL), NaOH, HCl, potassium ferrocyanide, potassium ferricyanide, Tris-HCl and the other chemicals used in this project were purchased from Sigma. A Tris buffer solution (TBS) with a concentration of 0.05 M was prepared in a doubly distilled water (DDW) and its pH was adjusted at 7.4 by the use of NaOH and HCl. 50 mg Gr and 40 µL IL were added to 0.5 mL ethanol and ultrasonicated for 30 min to obtain the Gr-IL. Standard solutions of CAT (0.05 M) and ASC (0.05 M) were prepared in the TBS (0.05 M, pH 7.4) and covered and kept in a refrigerator. An electrochemical probe solution, $[Fe(CN)_6]^{3-/4-}$ (0.05 M), was prepared from potassium ferrocyanide and potassium ferricyanide in 0.05 M KCl and used for some of our electrochemical experiments.

2.2. Instruments and software

A 302 N-high performance Autolab which has been purchased from Metrohm-Autolab Company was available in our laboratory and used for performing electrochemical experiments. This instrument was equipped with an electrochemical cell having three electrodes including a bare glassy carbon electrode (GCE), a Pt wire and an Ag/AgCl electrode as working, counter and reference electrode, respectively. The Autolab was controlled by the Nova software. A FEI Quanta 450 scanning electron microscope was used to take scanning electron microscopic (SEM) images. An ELMERON pH-meter was used to adjust the pH of the solutions. Multivariate curve resolution-alternating least squares (MCR-ALS) was run in MATLAB (Version 7.5). SPECFIT [25], SQUAD [26], EQUISPEC [27] and REACTLAB [28] were available in archive of our laboratory. Molegro virtual docker

(MVD) and LigPlus were used for molecular modeling studies. ChemBioOffice was used to generate PDB file of the ASC and the PDB file of the CAT was downloaded from protein data bank.

2.3. Electrochemical experiments

All the electrochemical experiments were performed with the help of the Autolab mentioned in the previous section. In all experiments related to the investigation of the binding of CAT with ASC, a known concentration of ASC was poured in the electrochemical cell and CAT was gradually added to it until its final concentration would be equal with ASC concentration. Each solution was stirred (1000 rpm) for 15 s and then, its electrochemical response was recorded. All the experiments were performed at room temperature.

2.4. Foundations used for hard- and soft-modeling of the data

Prior to any hard- or soft-modeling of the data, number of the components was determined by principal component analysis (PCA). Hard-modeling of the data was performed by SPECFIT, SQUAD, EQUISPEC and REACTLAB by defining suitable models according to the results of the PCA and an initial estimation of the binding constant (K_b) and stoichiometry of the complex species (CAT-ASC_n) obtained by experimental results. Soft-modeling of the data was performed by MCR-ALS. For more understanding about details of the algorithms mentioned above, the reader is referred to study Refs. [25-28].

3. Results and discussion

In this work, we are going to do a project in which binding and inhibition of the CAT by ASC will be investigated at the surface of the GCE. Structures of CAT and ASC are shown in Fig. 1. The ASC is an electroactive substance and investigation of the enzyme inhibition by electrochemical methods are interesting therefore, we are going to use electrochemical methods to investigate binding and inhibition of the CAT by ASC which will be expanded in next sections.

Fig. 1

3.1. Characterization of the modifications applied to the GCE

All the studies related to the investigation of biding of CAT and ASC have been performed at the surface of the GCE but, the final step of this work has been devoted to develop a novel electroanalytical method based on CAT-ASC interactions for determination of the CAT. In order to increase the sensitivity of the developed method, we

have modified the GCE with Gr-IL. Therefore, this section of our study has been devoted to the characterization of the modifications applied to the GCE. Observation of the electrode surface by SEM is a useful technique which can help us to see the modifications at the GCE surface. Fig. 2 shows the SEM image taken from the surface of the Gr-IL/GCE. The SEM image taken from the surface of the GCE is shown as the inset of Fig. 2 and as it can be seen, the GCE has a smooth surface and after its modification with Gr-IL, the Gr sheets could be observed at its surface.

Fig. 2

Electrochemical techniques such as cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) are very useful for characterization of the modifications applied to an electrode. Therefore, these techniques have been used to characterize the modifications applied to the GCE and the results are shown in Fig. 3 which confirmed that the GCE showed a well-defined CV (Fig. 3A, curve *a*) which was clearly improved by its modification with Gr-IL (Fig. 3A, curve *b*). An EIS response has a semicircle portion whose diameter is proportional to the charge transfer resistance (R_{ct}) at the electrode surface [29]. After modification of the GCE with Gr-IL, the R_{ct} was decreased which was related to the presence of the Gr-IL at the electrode surface. Therefore, the EIS and CV results were compatible and both of them confirmed the modification applied to the GCE to fabricate the sensing platform.

Fig. 3

3.2. Electrochemical behavior of the ASC *at the surface of the GCE*

In order to clarify the electrochemical behavior of the ASC at the GCE surface as the sensing platform which was used to investigate binding of ASC with CAT, CVs of the ASC at the surface of the GCE were recorded at different scan rates which are shown in Fig. 4A. In order to build a graphical representation, the CVs′ currents were regressed on scan rates as shown in Fig. 4B. As it can be seen, the currents are linearly correlated with scan rates which confirmed that the electrochemical behavior of the ASC at the GCE surface is governed by adsorption [29].

Fig. 4

3.3. Electrochemical investigation of the binding of ASC with CAT

We thought that the electrochemical techniques such as CV, EIS, differential pulse voltammetry (DPV), normal pulse voltammetry (NPV) and linear sweep voltammetry (LSV) could help us to obtain some information about binding and inhibition of the CAT by ASC. Therefore, we have performed some electrochemical experiments where a known concentration of the ASC was chosen and CAT was added to it and then, electrochemical responses were recorded. Electrochemical data obtained from different electrochemical experiments are shown in Fig. 5. As it could be seen, CV, LSV, DPV and NPV data confirmed that by the addition of the CAT to ASC, the peak intensities were decreased and shifted to more positive potentials which may be related to the change in environment around the ASC molecules upon interaction and binding with CAT [30,31]. The EIS data showed that with the addition of the CAT to ASC, the R_{ct} was increasing which confirmed more electron transfer resistance was occurred at the electrode surface. By binding of CAT with ASC and forming a complex, a bulky species is formed which can hinder the electrons to reach the electrode surface in some extend therefore, the R_{ct} is increased. As it could be seen, voltammetric and impedimetric data was analyzed by direct observation of them which gave us some initial information about binding of CAT with ASC, but we think that the data could be more processed to extract more information.

In order to determine stoichiometry (n) of the complex species (CAT-ASC_n) formed upon interactions of CAT with ASC, we have used mole ratio plots which are shown in Fig. 6. As it can be seen, all the plots are inflected at [CAT]/[ASC]~0.5 which recommend n~2. This important result can help us to start the next studies.

Further attempts were performed to calculate K_b and n by supposing CAT-ASC_n as the complex species formed upon interactions of the CAT and ASC. New DPV data (not shown) which were related to the titration of 1×10^{-4} M CAT with ASC (0-5×10^{-4} M) were recorded and analyzed according to the following equations [32,33]:

$$\text{CAT} + \text{n ASC} \rightarrow \text{CAT-ASC}_\text{n} \qquad K_b = \frac{[CAT-\text{ASC}_n]}{[CAT][\text{ASC}]^n} \qquad \textbf{(1)}$$

$$log\left[\frac{\Delta I}{(\Delta I_{max}-\Delta I)}\right] = n\ log\ K_b + n\ \log[\text{ASC}] \qquad \textbf{(2)}$$

where ΔI and ΔI_{max} refers to the current difference in the absence and presence of ASC and the maximum current, respectively. By regression of $log\left[\frac{\Delta I}{(\Delta I_{max}-\Delta I)}\right]$ on *log* [ASC], we could compute n and the K_b which were 2 and 4.98×10^5 mol^{-1} L, respectively. We performed all the actions which were required for conventional analysis of the voltammetric data, but we will expand our study by assisting it with chemometric methods to extract more information which will be described in next sections.

Fig. 5
Fig. 6

3.4. Chemometric assisted-electrochemical investigation of the binding of CAT with CYP

Here, we are going to perform some chemometric analyses of the electrochemical data with the aim of extracting new information which could help us to better justification of binding and inhibition of the CAT by ASC. The details of this type of study will be given in next sub-sections.

3.4.1. Determination of the number of the species involved in the system

In order to determine the number of the species involved in the system under our study, we have analyzed the CV, LSV, NPV and DPV data by PCA and the results are shown in Fig. 7. As it can be seen, all types of data showed three main components which may be related to free CAT, free ASC and one complex species.

Fig. 7

3.4.2. Hard-modeling of the data

Here, in order to examine the results of the experimental sections related to the calculation of the K_b, the CV, LSV, DPV and NPV data were modeled by several hard-modeling algorithms such as SPECFIT, SQUAD, EQUISPEC and REACTLAB and the results are collected in Table 1. As it can be seen, there is a good agreement between different algorithms and also with the results obtained by the analysis of the electrochemical data by the use of conventional methods. Hard-modeling of the data could show us that there was a relatively strong binding of CAT with ASC therefore, ASC could inhibit the CAT.

Table 1

3.4.3. Soft-modeling of the data

Here, we have modeled the data in a different way from the previous section to obtain some information to support the previous results and also to obtain new results which could help us to better understand binding and inhibition of the CAT by ASC [34]. To achieve these goals, CV, LSV, NPV and DPV data were row-wise augmented in a matrix which was used as the input of MCR-ALS. Resolution of the augmented matrix was started by performing singular value decomposition on it which showed us three main components which may be related to free CAT, free ASC and one complex species. Then, evolving factor analysis in forward and reversed directions was performed on the data which gave us an initial estimation of the concentration profiles. In next step, we did our best to define suitable constraints during ALS optimization which were including application of the non-negativity constraint to the concentrations. By starting the ALS optimization, we could obtain the optimized concentration profiles as shown in Fig. S1. As it can be seen, concentration of the ASC is decreasing upon addition of the CAT to it and a new species is forming which is related to the complex species and the curve which is related to the complex species reaches to a maximum at [CAT]/[ASC]~0.5 which confirms that the complex species is CAT-ASC_2. Therefore, results of this section showed us the variations in concentration of the species involved in the system under our study and also confirmed the previous results related to determination of the stoichiometry of the complex species.

As can be seen, electrochemical methods in combination with chemometric methods could help us to design an efficient method which will be more useful to extract useful information [35-46].

3.5. Molecular docking

Here, we are going to use molecular docking methods to theoretically investigate binding of ASC with CAT and to achieve this goal, the ASC was docked with CAT by the MVD software and the results are shown in Fig. S2A. As it can be seen, Val 383, Asn 385, Gln 387, Arg 388, Asp 389, Asn 397, Cys 393, Asn 369, Gln 398 and His 372 from CAT are interacted with ASC. The outputs showed that the K_b and ΔG were 4.65×10^5 mol^{-1} L and -28.33 kJ mol^{-1}, respectively. In order to the analysis types of the interactions of the residues of the CAT with ASC, the output of the molecular docking method was analyzed by LigPlus and the results are shown in Fig. S2B. As it can be seen, Gln 398, His 372 and Asn 385 have formed three hydrogen bonds with ASC while Arg 388,

Asn 369, Val 383 and Gln 387 were hydrophobically interacted with ASC. Therefore, the results of this section not only confirmed the previous results but also gave us new information to have a deeper insight to the binding of ASC with CAT. Binding of ASC with CAT is a relatively strong binding which confirms inhibition of the CAT by ASC.

3.6. Developing an electroanalytical method based on investigated interactions

In this section, we have developed a novel impedimetric method based on CAT-ASC interactions for determination of the CAT. In order to increase the sensitivity of the developed method, the Gr-IL/GCE was used as the sensing platform. Here, the Gr-IL/GCE was immersed into 1 μM ASC and CAT was added to it in the range of 1-50 nM and the EIS responses were recorded which are shown in Fig. S3A. As it can be seen, the sensor response was continuously changed by the addition of the CAT to ASC. In order to calibrate the sensor response, the R_{ct} values were regressed on concentrations of the CAT as is shown in Fig. S3B. The sensitivity of the developed method was calculated to be 23.4 Ω nM^{-1} while detection limit of the developed method was calculated according to $3S_b/m$ (S_b is the standard deviation of the blank and m is the slope of the calibration graph) which was 0.5 nM. Long-term stability of the sensor response was examined by its application to the determination of 20 nM CAT during six weeks and our results showed that the sensor was able to retain 96% of its original response, Fig. S3C. Repeatability of the sensor response was examined by its application to the determination of 20 nM CAT for 8 times and our results confirmed that the relative standard deviation (*RSD*) was 2.38% which confirmed that the senor response was repeatable. In order to examine the reproducibility of the sensor, six sensors were fabricated and they were applied to the determination of 20 nM CAT. The results (not shown) confirmed that the *RSD* was 3.35% which guaranteed the reproducibility of the sensor response.

4. Conclusions

By this article, we have collected results of an interesting study on binding and inhibition of the CAT by ASC in which different types of electrochemical data including CV, LSV, DPV and NPV were recorded and analyzed by conventional and chemometric methods. Both of the methods confirmed formation of the CAT-ASC_2 as a complex species. Our results confirmed formation of three main components which were related to free, CAT,

free ASC and CAT-ASC_2 as a complex species. Chemometric hard- and soft-modeling methods gave us quantitative and qualitative information, respectively. Our study was also more expanded by the use of molecular docking methods which could help us to have a better insight to the mentioned interactions. Finally, an impedimetric method based on CAT-ASC interactions was developed to introduce a novel method for determination of the CAT.

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Caption to figures:

Fig. 1. Molecular structure of (A) CAT and (B) ASC.

Fig. 2. The SEM images of the Gr-IL/GCE and the insert shows the SEM image taken from the surface of the GCE.

Fig. 3. (A) CVs and (B) EISs of GCE (a) and Gr-IL/GCE (b) in $[Fe(CN)_6]^{3-/4-}$ (0.05 M). Scan rate of the CVs was 0.05 V s^{-1}.

Fig. 4. (A) CVs of 1×10^{-4} M ASC recorded at the surface of the GCE at different scan rates ranging in 0.1-1 V s^{-1}, (B) regression of the CVs′ currents on scan rates to clarify the electrochemical behavior of the ASC at the GCE surface.

Fig. 5. (A) CVs, (B) LSVs, (C) NPVs, (D) DPVs and (E) EISs of 1×10^{-4} M ASC in the presence of addition of the CAT in the range of 0-1×10^{-4} M. The EISs were recorded in the presence of 0.05 M $[Fe(CN)_6]^{3-/4-}$. All the data have been recorded at the surface of the GCE.

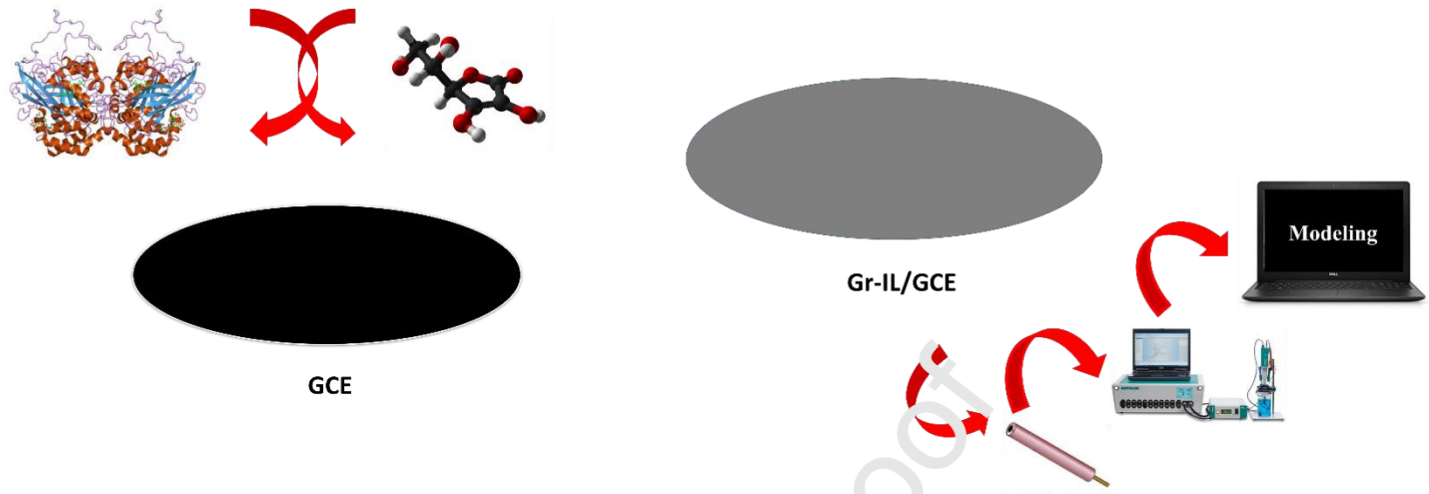
Fig. 6. Mole-ratio plots obtained from (A) CV, (B) LSV, (C) NPV and (D) DPV data by depicting currents versus [CAT]/[ASC].

Fig. 7. Results of performing PCA on (A) CV, (B) LSV, (C) NPV and (D) DPV data.

Scheme 1. Schematic representation of the steps of the study described in this article.

Table 1. Hard-modeling of the electrochemical data for computing K_b.

Algorithm	SQUQD	SPECFIT	EQUISPEC	REACTLAB
DPV data	$4.33(\pm0.04)\times10^5$	$4.54\ (\pm0.06)\times10^5$	$4.76\ (\pm0.09)\times10^5$	$4.21\ (\pm0.05)\times10^5$
CV data	$4.28(\pm0.06)\times10^5$	$4.87\ (\pm0.07)\times10^5$	$4.77\ (\pm0.07)\times10^5$	$4.24\ (\pm0.01)\times10^5$
LSV data	$4.74(\pm0.05)\times10^5$	$4.34\ (\pm0.05)\times10^5$	$4.35\ (\pm0.05)\times10^5$	$4.64\ (\pm0.03)\times10^5$
NPV data	$4.66(\pm0.05)\times10^5$	$6.51\ (\pm0.05)\times10^5$	$4.41\ (\pm0.04)\times10^5$	$4.75\ (\pm0.05)\times10^5$



Scheme 1.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

All the authors contributed equally in performing all sections to do this project.