Bioelectrochemistry 80 (2011) 169–174

Contents lists available at ScienceDirect

Bioelectrochemistry

journal homepage: www.elsevier.com/locate/bioelechem

Amperometric sensor for hydrogen peroxide based on direct electron transfer of *spinach* ferredoxin on Au electrode

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ARTICLE INFO

Article history:
Received 16 April 2010
Received in revised form 9 August 2010
Accepted 19 August 2010
Available online 26 August 2010

Keywords:
Ferredoxin
Cyclic voltammetry
Biosensor
Nanobiochip
Hydrogen peroxide detection

ABSTRACT

A protein-based electrochemical sensor for hydrogen peroxide (H_2O_2) was developed by an easy and effective film fabrication method where *spinach* ferredoxin (Fdx) containing [2Fe–2S] metal center was cross linked with 11-mercaptoundecanoic acid (MUA) on a gold (Au) surface. The surface morphology of Fdx molecules on Au electrodes was investigated by atomic force microscopy (AFM). Electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) were employed to study the electrochemical behavior of adsorbed Fdx on Au. The interfacial properties of the modified electrode were evaluated in the presence of $Fe(CN)_6^{3-/4-}$ redox couple as a probe. From CV, a pair of well-defined and quasi-reversible redox peaks of Fdx was obtained in 10 mM, pH 7.0 Tris–HCl buffer solution at −170 and −120 mV respectively. One electron reduction of the $[2Fe\text{-}2S]^{2+}$ cluster occurs at one of the iron atoms to give the reduced $[2Fe\text{-}2S]^{+}$. The formal reduction potential of Fdx ca. −150 mV (vs. Ag/AgCl electrode) at pH 7.0. The electron-transfer rate constant, k_s, for electron transfer between the Au electrode and Fdx was estimated to be 0.12 s^{-1}. From the electrochemical experiments, it is observed that Fdx/MUA/Au promoted direct electron transfer between Fdx and electrode and it catalyzes the reduction of H_2O_2. The Fdx/MUA/Au electrode displays a linear increase in amperometric current for increasing concentration of H_2O_2. The sensor calibration plot was linear with $r^2 = 0.998$ with sensitivity approximately 68.24 μAm M^{-1} cm^{-2}. Further, the effect of nitrite on the developed sensor was examined which does not interfere with the detection of H_2O_2. Finally, the addition of H_2O_2 on MUA/Au electrode was observed which has no effect on amperometric current.

1. Introduction

The construction of protein-based thin films on electrode surfaces has attracted considerable interest in the development of novel biosensors as well as in the fundamental research of protein properties, and its electron-transfer reactions [1,2]. The determination of hydrogen peroxide (H_2O_2) using these electrodes is of great importance because H_2O_2 plays an important role in food, pharmaceutical, and environmental analysis [3]. H_2O_2 can be detected by using any enzymatic material as the electrocatalyst for electrochemical reduction.

Direct electron transfer between biomolecules and electrode has been extensively studied because of its importance in fundamental science in biological reactions [4], and broad applications in enzyme fuel cells [5], bioelectronics [6], and biosensors [7,8]. Enzyme-based biosensors are usually fabricated by immobilizing enzymes on various types of electrodes or modified electrodes. Since the catalytic process and chemical properties of redox enzymes are very complex, utilization of the direct electrochemistry of redox enzymes has attracted much interest. Better understanding of the electron-transfer mechanism and the structure of redox enzymes, and the development of mediator-free biosensors have been realized [9]. The direct electrochemistry of enzyme refers to the direct electron communication between the electrode and the active center of the enzyme without the participation of mediators or other reagents.

Several researchers proposed various methodologies to detect organic/inorganic materials. In our previous research we proposed some techniques to detect the biological materials and bioeletronic device with simple function [10–15]. Mainly, for detection of H_2O_2, commonly horseradish peroxidase (HRP) [16] and hemoglobin (Hb) [17] have been widely used to construct amperometric biosensors for the determination of H_2O_2 [18,19] for the sensing of glucose [20] and amino acids [21]. These heme proteins serve biochemical roles involving electron transfer, oxygen transfer and storage, and metabolism. Hemoglobin, which is a redox active protein consisting of four electroactive-iron (III) hemes as prosthetic groups enables reversible conversion of Hb-Fe(III) to Hb-Fe(II) which is responsible for its electroactivity. This Hb has disadvantages, as the rate of

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doi:10.1016/j.bioelechem.2010.08.002

electron transfer from the protein to the surface of the electrodes modified directly by hemoglobin is slow; moreover a large, three-dimensional structure of hemoglobin leads to inaccessibility of the redox centers that are located inside the protein. Therefore, direct electron transfer between the hemoglobin and electrode is difficult [22]. Intensification of electron-transfer rate between hemoglobin and electrode surface can be achieved using electro-mediators [23,24]. There are numerous methodologies where biofunctionalized nanomaterials such as carbon nanotubes [25,26], enzyme with nanoparticles [27] are used for H_2O_2 detection. Here, in the present research we select *spinach* ferredoxin to check the catalytic response to H_2O_2.

Ferredoxins are iron-sulfur proteins that function as electron carriers in many metabolic processes. *Spinach* ferredoxin contains clusters of two iron atoms and represented as $[2Fe\text{-}2S]^{2+}$. The total number of amino acid residues (AA) that Fdx contains is 97 which have a molecular weight ranging from 11,000 to 11,900 [28]. The Fe (III) ions in $[2Fe\text{-}2S]^{2+}$ are bridged by two sulfides and the tetrahedral coordination of each iron completed by cysteine residues. One electron reduction of the $[2Fe\text{-}2S]^{2+}$ cluster occurs at one of the iron atoms to give the reduced $[2Fe\text{-}2S]^{+}$ cluster. In the oxidized state, both iron atoms are in a similar chemical state, which appears from the chemical shift and quadrupole splitting to be high-spin Fe^{3+}. In the reduced state the iron atoms are different and the molecule appears to contain one high-spin Fe^{2+} and one high-spin Fe^{3+} atom. Fig. 1 illustrates the model envisages for the iron-sulphur spinach ferredoxins. In the oxidized state the two high-spin ferric iron atoms where only one of these iron atoms accepts an electron on reduction. The spins of the iron atoms are coupled in such a way that there is no net spin in the ground state ($S = 0$). When the ferredoxin is reduced one electron is transferred to the specific iron atom, which then becomes high-spin ferrous; thus in the reduced state there is one high-spin ferrous and one high-spin ferric iron atom. The spins on the iron atoms are still coupled, but there is a net spin $S = ½$ [29].

The Fdx was cross linked with 11-mercaptoundecanoic acid (MUA) on Au electrode and studied to develop an amperometric H_2O_2 sensor. The determination of H_2O_2 is performed at low potential in a deoxygenated Tris–HCl buffer solution. When Fdx catalyzes a substrate, such as H_2O_2, the active site form of Fdx is from Fe (III) to Fe (II) at the electrode. The direct electron transfer [30–32] has been achieved by immobilizing Fdx on MUA modified Au electrode.

In the present study, Fdx film on Au was selected and used to study the amperometric response for detection of H_2O_2. Atomic force microscopy, cyclic voltammetry and electrochemical impedance spectroscopy have been employed to characterize the prepared electrodes. The Fdx immobilized electrode has been realized for good electron transfer between Fdx and Au. The developed sensor exhibits good electrocatalytic properties for the electrochemical reduction of hydrogen peroxide. Also, the effect of other reducible compunds such as nitrite was checked and further the results were compared with catalytic response of MUA/Au electrode.

2. Experimental

2.1. Materials and reagents

Ferredoxin from spinach was purchased from Sigma, used as received and stored at −20 °C. 11-mercaptoundecanoic acid (HS $(CH_2)_{10}COOH$, MUA, Aldrich) was used as received without any further purification. N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-diethylamino-propyl) carbodiimide hydrochloride (EDC) was obtained from Sigma. The MUA deposition solution was prepared by dissolving MUA in absolute ethanol and its final concentration was 30 mM. 30% hydrogen peroxide was brought form Daejung materials and chemicals (South Korea) and diluted in DI water to prepare solutions. The electrolyte for CV experiments is 10 mM Trizma® hydrochloride solution of pH 7.0 purchased from Sigma-Aldrich, and for NHS and EDC molar solutions, ethanol/water solution was used. All other chemicals were of reagent grade and used as received. All solutions were prepared with pure water (18 MΩ cm) purified with a Milli-Q system (Millipore, Bedford, MA). All electrochemical experiments were performed in 10 mM Tris–HCl buffer solution with pH 7.0. High purity nitrogen and Argon were used for deaeration. The buffer and sample solutions were purged with highly purified nitrogen for at least 10 min prior to each experiment. Nitrogen atmosphere was maintained over the solutions during experiments.

2.2. Formation of the self-assembled monolayer (SAM) on gold

First of all, Au working electrodes of 0.25 cm^2 was kept in piranha solution (1:3) v/v, 30% H_2O_2 and concentrated H_2SO_4 for 4.0 min and rinsed thoroughly with distilled water. After drying with nitrogen gas, the clean electrode was immediately immersed in the MUA deposition for 1 h at room temperature, followed by rinsing off the residual MUA molecules with ethanol. Then the self-assembled electrode was soaked in 1 mL of 100 mM EDC and 50 mM NHS for 1 h to activate the COOH-terminated group of MUA [33]. Afterwards, 20 μL of 0.1 mg/mL Fdx in pH 7.4 Tris-HCl was added onto the activated MUA self-assembled layer for 2 h, it is then cleaned with distilled water. The electrode modification process for Fdx immobilization on Au electrode was represented in schematic diagram as shown in Fig. 2.

2.3. Surface characterization with atomic force microscopy (AFM)

The morphology of the bare Au surface and Fdx immobilized Au surface was investigated by Atomic force microscopy (AFM) (Nanoscope IV/Multimode, Digital Instruments) equipped with a 100 μm scanner operated in tapping mode. The probes with 1-100 Ω cm phosphorous (n) doped (Si) tip with a spring constant of 20-80 N/m, having resonant frequencies between 262 and 307 kHz were used.

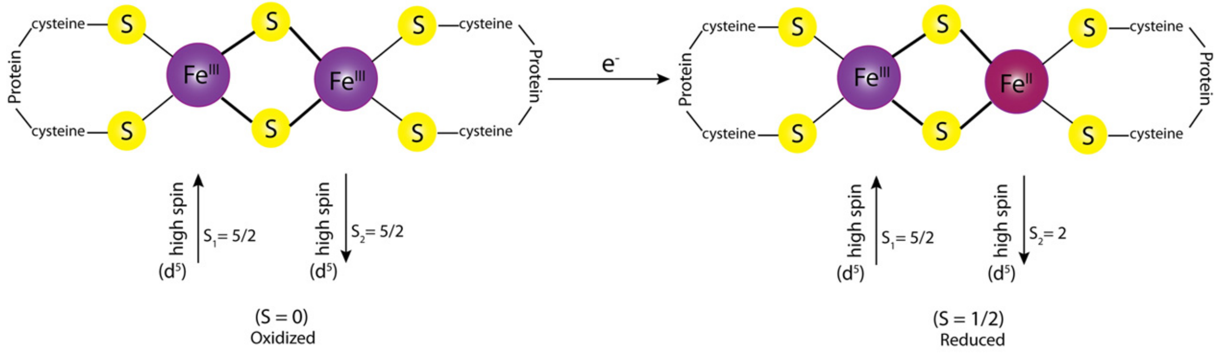

Fig. 1. Proposed model for electron transfer of the iron-sulphur group in spinach ferredoxin.