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Enzymatic Direct Electron Transfer at Ascorbate Oxidase-modified Gold Electrode Prepared by One-step Galvanostatic Method

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A one-step enzyme immobilization by galvanostatic method at gold electrode was applied to achieve direct electron transfer (DET) of ascorbate oxidase from *Acremonium sp*. (ASOM). The enzyme immobilization was optimized and ASOM was successfully deposited on the gold electrode surface, typically by flowing the oxidation current of 6 μA corresponding to oxygen evolution for 4 min, which results in a decrease in the local pH in the vicinity of the electrode surface and the precipitation of ASOM on the electrode surface due to the neutralization of its surface charge. A well-defined redox wave with a formal potential of 242 mV (vs. Ag | AgCl | $KCl_{(sat)}$) was observed in 5.0 mM phosphate buffer solution (pH 7.0), confirming DET between ASOM and gold electrode surface.

Keywords: Ascorbate oxidase, Direct electron transfer, Galvanostatic immobilization

1. INTRODUCTION

A direct electron transfer (DET) reaction between enzyme and electrode can yield an important information on the thermodynamics and kinetics of its biological redox processes. A variety of pioneering techniques have been utilized for the immobilization of enzymes on to electrode surface. Entrapment of enzymes into electro-deposited polymers of e.g., pyrrole and chitosan is well known[1-3]. It has been reported that electrodeposited enzyme forms an extensive and compact layer on the electrode surface[4]. Use of detergent such as Triton-X® above its critical micelle concentration (CMC) can enhance the electrochemical deposition of enzyme[5]. However, high concentration above CMC of detergent might denature the enzyme. Therefore, immobilization of enzymes in the absence of

detergent is of great interest to fabricate bioelectrodes for biosensors and biofuel cells. Among the various procedures, galvanostatic immobilization is attractive since it is a one-step method. This method has been used to immobilize various enzymes such as glucose oxidase[5,6], lactate oxidase[7], glutamate oxidase[7], arsenite oxidase[8] and lysozyme[4] on electrode surface.

Multicopper oxidases (e.g., laccase, bilirubin oxidase and ascorbate oxidase) are the blue copper proteins having the potential of four-electron oxygen reduction to water by sequential electron uptake from a reducing substrate such as phenols and ascorbate[9-13]. Numerous studies have been reported on the DET of laccase[14-22] and bilirubin oxidase[23-33]. However, there are only a few reports on the DET of ascorbate oxidase[34-36]. Sakurai first reported the DET of cucumber ascorbate oxidase at Au electrodes modified with various promoters (bis(4-pyridyl) disulphide, bis(2-aminoethyl) disulphide, 3,3'-dithiodipropionic acid and diphenyl disulphide)[34]. Santucci and co-workers confirmed the possibility of DET between a gold electrode and ascorbate oxidase using ascorbate oxidase embedded within a polymeric film of an anionic exchange resin containing tributylmethyl phosphonium chloride (TBMPC) bound to polystyrene, cross-linked with divinylbenzene[35]. Recently, Murata et. al. have found that a DET of ascorbate oxidase from *Acremonium sp.* HI-25 (ASOM) on a gold electrode is facilitated by a TBMPC membrane and MPA-SAM (3-mercaptopropionic acid self-assembled monolayer) combined system[36]. It has been reported that ascorbate oxidase does not show a direct electrochemistry with graphite surface[37].

In this study, one-step galvanostatic immobilization of ASOM on the gold electrode surface is attempted with a view to achieving its DET. ASOM immobilization was achieved by the precipitation of ASOM on the gold electrode surface owing to a decrease in local pH value due to the electrolytic oxygen evolution and its DET was realized successfully.

2. EXPERIMENTAL

2.1. Materials and instrumentation

Ascorbate oxidase from *Acremonium sp.* (T-53) was purchased from ASAHI KASEI PHARMA (Japan). All other chemicals were purchased from WAKO Pure Chemical Industries, Ltd. and were used as received. All the solutions were prepared using deionized water (18 MΩ-cm) purified by Milli-Q water purification system (Millipore, Japan).

Galvanostatic deposition of ASOM onto gold electrode was performed using a potentiostat/galvanostat (PS-07, Toho Technical Research, Japan). Cyclic voltammetric measurements were performed in phosphate buffer solution (PBS, 5.0 mM, pH 7.0) using an electrochemical analyzer (ALSCHI 760D, CHI instruments) and a conventional two-compartment three-electrode electrochemical cell, where Pt wire and $Ag \mid AgCl \mid KCl_{(sat.)}$ electrodes were used as the counter and reference electrodes (C.E. and R.E.), respectively. Gold electrode (Bioanalytical Systems Inc. (BAS);1.6 mm in diameter) served as the working electrode (W.E.). Electrolyte solutions were deaerated by bubbling N_2 gas into the solution for at least 30 min prior to each electrochemical

measurement. All the measurements were carried out at room temperature (25±1˚C). The apo enzyme of ASOM (Apo-ASOM) was prepared according to the reference[38].

2.2. Cleaning and pretreatment of gold electrode

Prior to use, gold electrode (AuE) was polished first with fine emery paper (#2000, SANKYO, Japan), and then with aqueous slurries of fine alumina powder (particle sizes: 1.0 and 0.06 μm) with the help of a polishing microcloth. To remove the residual alumina particles the polished electrode was ultrasonicated in Milli-Q water for 10 min. Finally, the electrode surface was electrochemically pretreated in 0.1 M H_2SO_4 solution by successive and multiple potential cycling between -0.2 and +1.5 V vs. Ag | AgCl | $KCl_{(sat.)}$ at 500 mV/s, until the reproducible characteristic cyclic voltammogram (CV) of AuE was obtained.

2.3. Galvanostatic immobilization of ASOM

The galvanostatic immobilization of ASOM on the AuE was carried out in PBS (5.0 mM, pH 7.0) containing 50 μM ASOM by controlling the magnitude of the applied current and the electrolysis time. The immobilization of ASOM is based on decrease in the local pH in the vicinity of the electrode surface due to the electrolytic oxidation of water to oxygen and the resulting neutralization of the surface of ASOM (pI = 4)[39], which causes the precipitation of ASOM on the AuE surface.

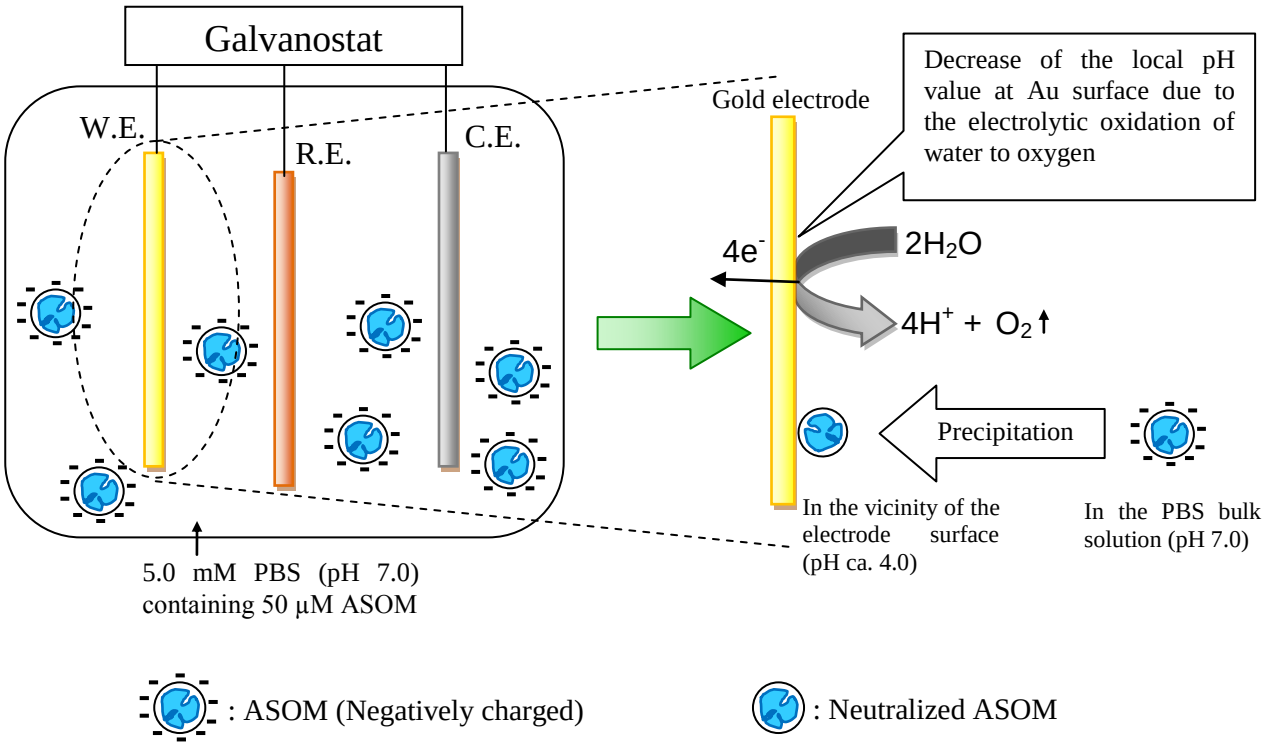

Figure 1. Schematic representation of galvanostatic immobilization of ASOM.

A schematic representation of galvanostatic immobilization of ASOM is presented in Fig.1. Thus prepared electrode was washed with PBS (5.0 mM, pH 7.0) and referred as ASOM-immobilized AuE. Similarly, Apo-ASOM was also immobilized on the AuE (designated as Apo-ASOM-immobilized AuE).

3. RESULTS AND DISCUSSION

Figure 2 shows the dependences of the surface coverage (Γ) of ASOM adsorbed on the AuE surface by the galvanostatic immobilization upon the applied current and the duration, in which the Γ values were evaluated from the well-defined CVs corresponding to the redox reaction (i.e. DET) of the adsorbed ASOM (shown below) using the following equitation[40]:

$$\Gamma = Q / nFA \qquad (1)$$

where Q is the amount of charge calculated by the integration of the anodic peak current for the oxidation of the adsorbed ASOM (corrected for the background current), n the number of electrons involved in the redox reaction (assumed as $n = 1$), F the faraday constant and A the geometric electrode area (0.020 cm^2).

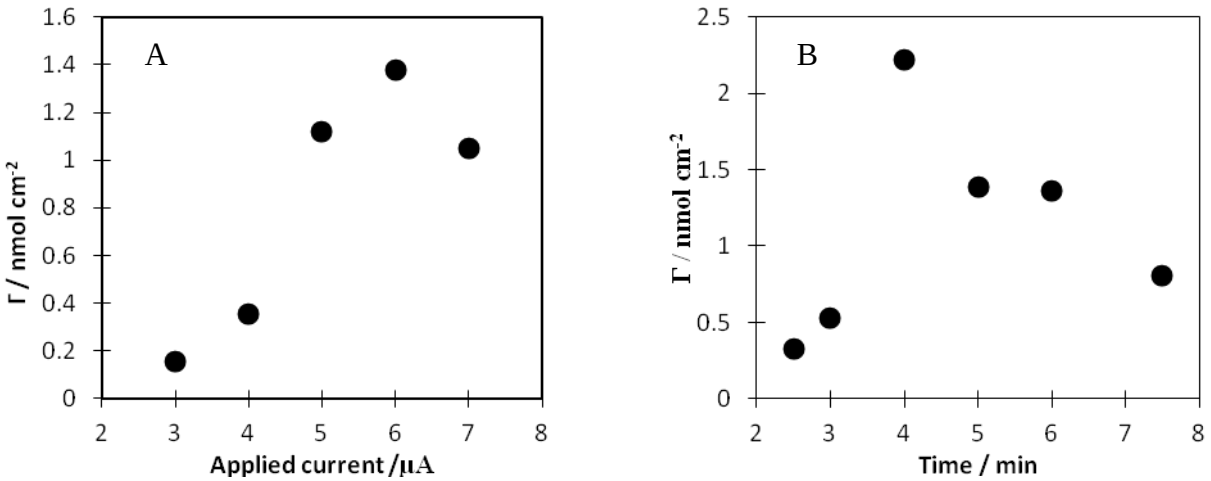

Figure 2. Surface coverage (Γ) of ASOM immobilized on the AuE as a function of (A) the applied current at a constant electrolysis time (5 min) and (B) the electrolysis time at a constant current of 6 μA.

It is expected that as the applied current at a constant duration increases or the duration at a constant current increases, the local pH in the vicinity of the AuE surface decreases gradually due to the electrolytic oxygen evolution (typically $2H_2O - 4e^- \longrightarrow 4H^+ + O_2$), which may result in the neutralization of the ASOM surface around pH 4 because of its pI = 4 and the consequent precipitation

(adsorption) on the electrode surface. In conformity with this view, the Γ values were found to increase with increasing the applied current at a constant duration and the duration at a constant applied current, i.e., up to 6 μA at 5 min duration and up to 4 min at the applied current of 6 μA. In this case, the electrode potential was found to change from ca. 0.8 V (before the electrolysis) to 1.72 V when the 6 μA was applied for 4 min. Further increase in the applied current and the duration led to the decrease in the Γ values. It might be due to the fact that the local pH in the vicinity of the AuE surface becomes gradually lower than the pI value (ca. 4) of ASOM and consequently the net charge of ASOM surface becomes charged positively and it becomes dissolved, i.e., the lower is the local pH, the larger is the degree of the desorption from the AuE surface. Fig. 2 also demonstrates that it is possible to control the surface coverage of ASOM on the AuE by suitably tuning the magnitude of applied current and the duration. Shown in Fig. 3 are typical CVs obtained at bare AuE, AuE on which ASOM was adsorbed physically (abbreviated as ASOM-physically immobilized AuE), Apo-ASOM-immobilized AuE and ASOM-immobilized AuE in 5.0 mM PBS (pH 7.0), in which the Apo-ASOM and ASOM-immobilized AuEs were prepared by the above-mentioned galvanostatic immobilization in Apo-ASOM and ASOM solutions, respectively. ASOM-physically immobilized AuE was prepared by putting 4 μl of 50 μM ASOM solution on the bare AuE and drying it at room temperature. This physical adsorption of ASOM on the AuE did not give any redox response (voltammogram 2). Both bare AuE and Apo-ASOM-immobilized AuE also did not show redox responses (voltammograms 1 and 3). A well-defined redox wave was observed only when ASOM was immobilized galvanostatically as mentioned above (voltammogram 4). Increase in non-faradaic current after the immobilization of Apo-ASOM and ASOM indicates that they are deposited really on the AuE surface. A comparison of voltammograms 3 and 4 clearly demonstrates that the redox wave observed for the ASOM-immobilized AuE is ascribable to the DET of ASOM.

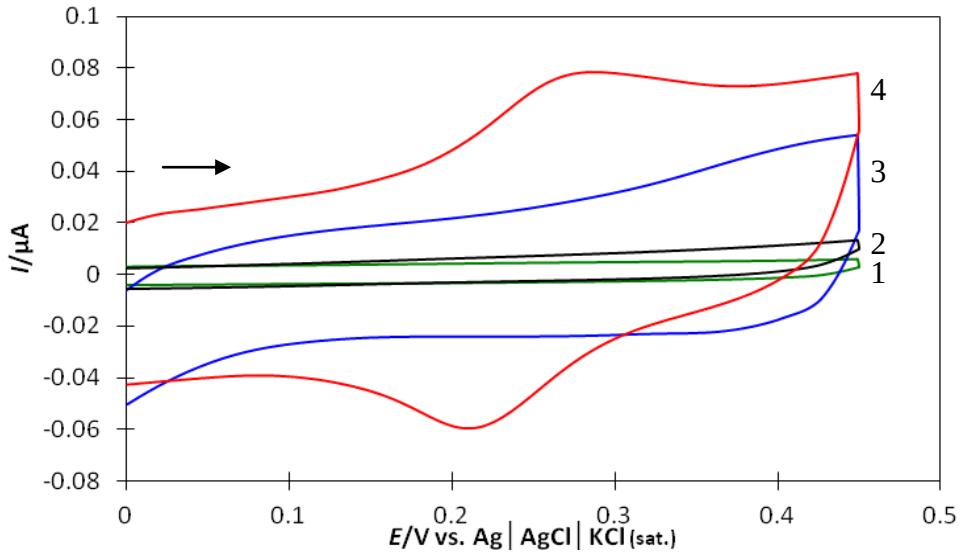

Figure 3. CVs obtained at bare AuE (1), ASOM-physically immobilized AuE (2), Apo-ASOM-immobilized AuE (3) and ASOM-immobilized AuE (4) in 5.0 mM PBS (pH 7.0) under N_2 gas atmosphere. v = 10 mV/s.

Figure 4A shows the potential scan rate (v) dependence of the redox response observed at the ASOM-immobilized AuE in 5.0 mM PBS (pH 7.0). The ratios of the anodic peak current to cathodic one (I_p^a / I_p^c) are close to unity in the examined range of v (1 - 50 mV s^{-1}).

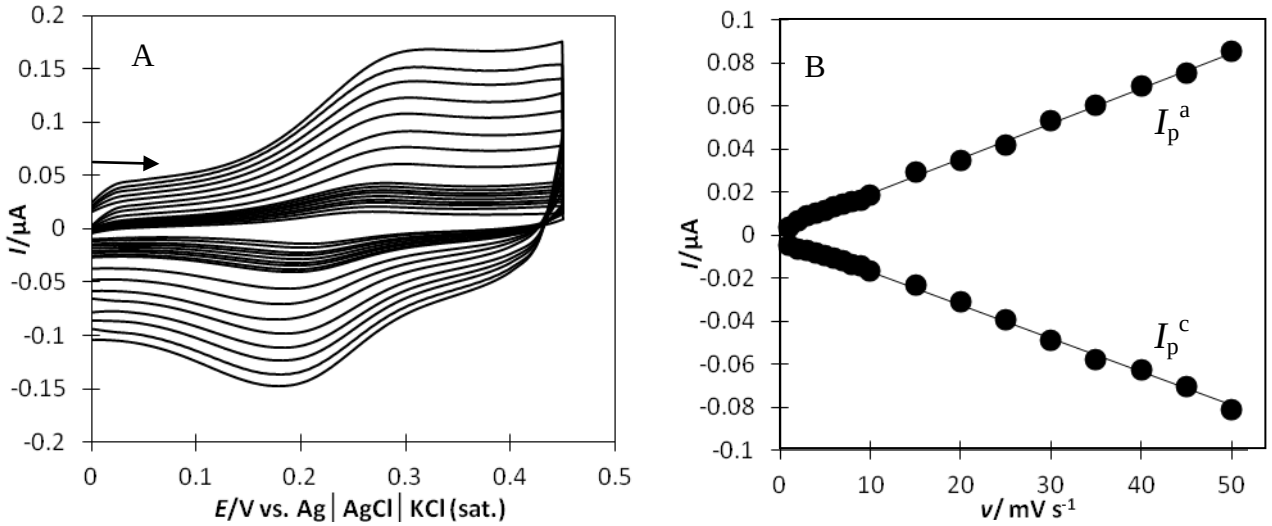

Figure 4. (A) CVs obtained for ASOM-immobilized AuE at different scan rates (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45 and 50 mV/s from inner to outer). (B) Plots of anodic and cathodic peak currents (background current-subtracted I_p^a and I_p^c, respectively) vs. scan rate (data were taken from Fig. 4A).

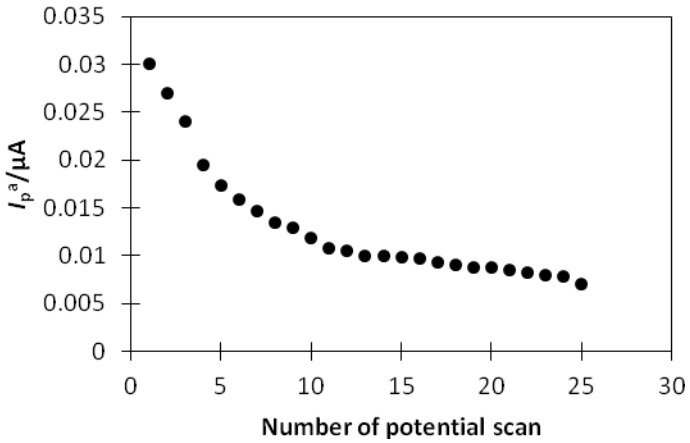

Figure 5. Plot of I_p^a vs. number of potential scan obtained at ASOM-immobilized AuE. Data were obtained by continuous potential cycling between 0 and 0.45 V in PBS(5.0 mM, pH 7.0) under N_2 atmosphere. v = 10 mV/s.

The I_p^a and I_p^c values are proportional to v, as expected for a surface-confined electrode process (Fig. 4B). The redox potential ($E^{\circ'}$), estimated as (E_p^a + E_p^c) / 2, where E_p^a and E_p^c are the anodic and cathodic peak potentials, respectively, is 242 ± 2 mV vs. Ag | AgCl | $KCl_{(sat.)}$ and ca. 40 mV more positive than the value measured by potentiometric titration for T1 Cu active site[41]. So far the

structure of ASOM is not known[42]. However, it has been reported that lactone ring of histidine near T1 site is exposed to the solution, while T2/T3 site is more deeply buried in case of the ascorbate oxidase from *Zucchini sp*.[12]. In addition, histidine-rich regions of ASOM have been reported to have a high sequence homology to the corresponding regions of other multicopper oxidases[42]. Thus we speculate that the observed redox response is ascribed to the redox reaction (i.e., DET) of the T1 site of ASOM. From the v dependence of ΔEp ($\equiv E_p^a - E_p^c$) at v = 10 - 200 mV s^{-1}, the electron transfer rate constant (k°) was estimated to be 0.3 ± 0.2 s^{-1}, assuming that the transfer coefficient (α) is 0.5, according to the Laviron's method[43].

Figure 5 shows the plot of I_p^a against the number of potential scan at 10 mV s^{-1} in the potential range between 0 and 0.45 V. As seen from this figure, I_p^a decreases gradually with the potential scan, i.e., after 25 cycles it decreased to 28% of its initial value. Thus the stability of ASOM should be further improved for its application, e.g., by covering the ASOM-immobilized AuE with a thin polymeric (electrolyte) film.

4. CONCLUSIONS

We have demonstrated that the one-step galvanostatic immobilization of ASOM is a simple, useful approach for realizing DET between ASOM and AuE. Furthermore, by tuning the magnitude of applied current and its duration, it is possible to control the active surface coverage of ASOM on the AuE. However, further stabilization of the ASOM layer is needed in order to fabricate ASOM-based bioelectrodes for biofuel cell and biosensor. Further work is underway for improving the stability and developing their application for ascorbic acid detection and electrocatalytic oxygen reduction. Deposition of other multicopper enzymes might be also possible using the present one-step galvanostatic procedure.

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