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Thermophilic biocathode with bilirubin oxidase from *Bacillus pumilus*

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ABSTRACT

Bilirubin oxidase (BOD) from *Bacillus pumilus* shows high thermostability in solution and high reactivity at high temperature for ABTS oxidation. When BOD is incorporated into a cross-linked redox-active hydrogel film consisting of pendant osmium moieties grafted on a polyvinylimidazole backbone on GC electrode, high catalytic current per enzyme molecule was achieved with redox mediator in a broad pH range from 4 to 7. The maximum current was increased with increasing the electrolyte temperature as high as 70 °C.

1. Introduction

An electrochemical oxygen reduction reaction (ORR) catalyzed by multicopper oxidase (MCO), such as laccase and bilirubin oxidase, typically proceeds under mild pH (from 3 to 7) and moderate temperature (20 to 40 °C), without releasing any intermediate, and at potential close to formal potential of oxygen/water [1–14]. Recently, CueO (copper efflux oxidase) from *Escherichia coli* has shown extremely high catalytic activity as electrocatalyst [15]. The major drawbacks of enzyme electrodes would be their lower activity and instability, compared to traditional metal electrocatalysts, especially, at high temperature above 50 °C. Only one laccase from *Bacillus subtilis* has been reported to operate efficiently in the 45–50 °C range [16,17]. Generally, it is difficult to improve the stability of an enzyme by protein engineering technologies without decreasing its activity. One very promising approach in this respect would be the screening of new MCOs. In our previous study, we identified a new BOD from the thermophilic bacteria, *Bacillus pumilus* [18], which was previously considered as a laccase [19]. Indeed this enzyme exhibited activity of oxidation toward bilirubin, which differentiate BODs from laccases. This BOD showed promising abilities for application in biofuel cells and implanted devices; good activity in solution at neutral pH and body temperature and good tolerance to the presence of NaCl and urate [18,20]. It was demonstrated by incorporating the new BOD in a redox hydrogel and comparing its performance with that of electrodes made with the more usual BOD from *Trachyderma tsunodae*. In particular, it permitted the construction of cathodes with increased lifetime. This new BOD coming from the thermophilic bacteria was active toward ABTS in acidic pH and at temperatures as high as 80 °C with very good stability, and then could represent an attractive alternative to platinum as a catalyst in rougher conditions. In this paper, we will present the temperature and pH dependence of the current response of BOD electrodes for ORR biocathodes based on mediated electron transfer (MET) reaction. The good performance of enzymatic electrodes at high temperature is demonstrated.

2. Experimental section

2.1. Chemicals and materials

BOD was produced and purified according to our previous paper [18]. Osmium polymer (PAA-PVI-[Os(4,4′-dichloro-2,2′-bipyridine)$_2$Cl$^-$]$^{+/2+}$) was synthesized as already published [21]. Poly(ethylene glycol) diglycidyl ether (PEGDGE 400) was purchased from Sigma-Aldrich. All other chemicals used were of reagent grade.

2.2. Temperature dependence of the enzymatic activity in solution

The enzyme activity profile as a function of the temperature was determined spectrophotometrically by the oxidation of ABTS at 420 nm ($\varepsilon_{420\ nm}$ = 36 mM^{-1} cm^{-1}) in a McIlvaine's citrate–phosphate buffer pH 4.0.

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2.3. Electrode preparation for MET reaction

The electrodes for the MET reaction were prepared by the reported three-step procedure. A deposition solution was prepared by mixing 3.0 μL of a 10 mg mL^{-1} aqueous redox polymer solution, 2.9 μL of milli-Q water, 1.2 μL of 5.0 mg mL^{-1} BOD in Tris–H_2SO_4 (pH 7.4, 50 mM) and 0.9 μL of 3.0 mg mL^{-1} PEGDGE in water. The mixed solution was pipetted onto the mounted hydrophilic GC electrode after the O_2 plasma treatment (1 Torr) for 10 min. The electrodes were cured for 18 h at room temperature before they were used. The amount of enzyme on the electrode was 6.0 μg.

2.4. Electrochemical measurements

Rotating disk cyclic voltammetry was performed on an electrochemical analyzer (CHI 842, CH-Instruments, USA). The electrode was rotated using a Pine instruments rotator. The counter and reference electrodes were a platinum wire and a Ag|AgCl electrode, respectively. The measurements were performed in a thermostated water jacket-equipped electrolysis cell. The electrolysis solution was either 0.1 M citrate buffer (pH 4–5) or 0.05 M phosphate buffer (pH 6–7) in a total volume of 100 mL. The scan rate was 10 mV s^{-1}.

3. Results and discussion

3.1. Temperature dependence of the enzymatic activity in solution

To evaluate the dependence of the activity of the purified BOD on the temperature, we measured its activity towards ABTS between 10 and 85 °C in a McIlvaine's citrate–phosphate 0.1 M buffer pH 4.0 (Fig. 1). As seen in Fig. 1, the relative activity increases upon increasing the temperature up to 75 °C and then levels off [18].

3.2. Temperature and pH dependence of the catalytic current based on the MET reaction

Fig. 2(a) shows the pH dependence of the current density obtained at pH 4, 5, 6, and 7. When ABTS is used as an electron donor for BOD, the relative catalytic activity of BOD at pH 7 was ca. 15% of that at pH 5 [18]. However, the catalytic limiting current density at pH 7 was one third of that at pH 5. The difference would be caused by complex factors; (1) the steric hindrance effect, (2) redox potential difference between an electron donating substrate and type 1 copper, and (3) the electrostatic interaction between the global charge of the mediator and the surface electrostatic potential near the type 1 copper site. To clarify this and improve the catalytic activity, the redox potential of the type 1 copper site at respective pH and 3D structure of BOD would be needed.

Fig. 2(b) shows the dependence of the current density on the temperature at pH 5 for a BOD modified electrode. The limiting current density increased upon increasing the temperature. The overall shape of the voltammograms is not significantly altered over the 37–70 °C temperature range. The percentages of the catalytic current densities at 37 and 50 °C relative to that at 70 °C are respectively 30 and 55%, which are in good agreement with those of the ABTS oxidation activity in solution as shown in Fig. 1. This clearly indicates that the limiting current density is determined by the enzymatic kinetics which depends on the temperature, not by the hydrogel structure, O_2 transportation rate, and O_2 concentration in the hydrogel. Although BOD from *T. tsunodae* modified hydrogel electrode showed high and stable catalytic activity at 37.5 °C, the catalytic current of the electrode increased with the temperature up to 40 °C and then declined [13]. While high current densities at temperature ~45–50 °C have been reported earlier for laccases [16,17], it has never been reported for BODs. At 70 °C, BOD from *T. tsunodae* loses all the current after ~3 or 4 min, however, the half-life time of BOD from *B. pumilus* modified electrode was 20 min (data not shown). The instability of the electrode might be due in part to the decomposition of the hydrogel network, since the activity of BOD from *B. pumilus* at 80 °C in a buffer solution had decreased by less than 50% at 90 min [18].

3.3. Electrochemical response of BOD using Os complex as a redox mediator at 70 °C

Catalytic performance of BOD and the redox polymer modified GC electrode in O_2-saturated buffer at 70 °C is represented in Fig. 3. The effect of mass transfer of O_2 dissolved in the buffer was examined by varying the rotation rate of the electrode. At low rotation rate, 500 rpm and 1000 rpm, a hump was observed between 0.3 and 0.4 V. This was attributed to the electrochemical response of a part of the Os complexes which did not work as a mediator in the MET reaction of BOD because of an insufficient supply of O_2 from the bulk solution. The current density at −0.1 V increased with the square root of the angular velocity ($\omega^{1/2}$), as shown in the inset of Fig. 3. The linear relation between the current density and $\omega^{1/2}$ held up to 2000 rpm, indicating that the electrode process is predominantly controlled by diffusion of O_2 to the hydrogel film. Diffusion controlled ORR currents catalyzed by

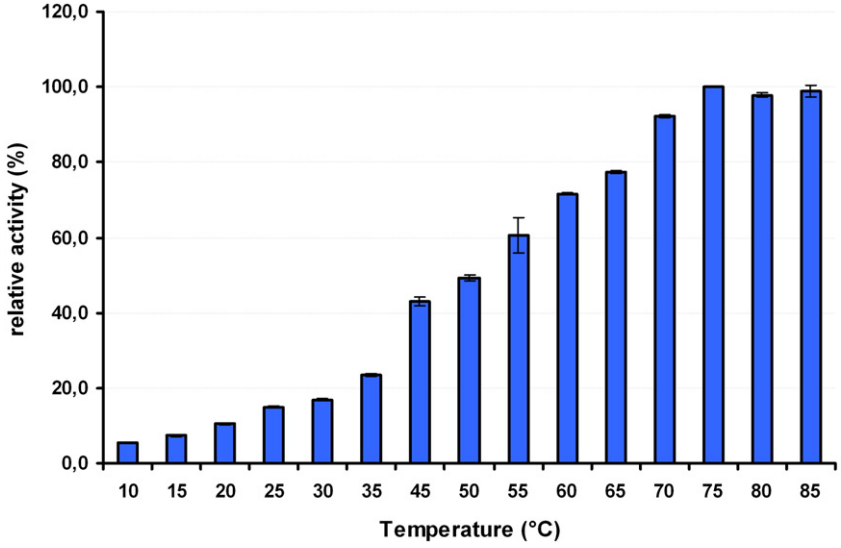

Fig. 1. Dependence of the enzyme activity on the temperature for the purified BOD from *Bacillus pumilus*. The activity was measured in a 0.1 M phosphate–citrate buffer pH 4 in the presence of 1 mM ABTS.