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Short communication

Development of a novel glucose enzyme fuel cell system employing protein engineered PQQ glucose dehydrogenase

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Abstract

Glucose dehydrogenase harboring pyrroloquinoline quinone as the prosthetic group (PQQGDH) from *Acinetobacter calcoaceticus* is an ideal enzyme for the anode of biofuel cell, because of its oxygen insensitivity and high catalytic efficiency. However, the application of PQQGDH for the bioanode is inherently limited because of its instability. Using Ser415Cys mutant whose stability was greatly improved, we constructed the biofuel cell system employing the engineered PQQGDH as the bioanode enzyme and bilirubin oxidase (BOD) as the biocathode, and compared the stability of the biofuel cell with that employing wild-type PQQGDH. The maximum power density was 17.6 μW/cm^2 at an external optimal load of 200 kΩ. Using Ser415Cys mutant, the lifetime of the biofuel cell system was greatly extended to 152 h, more than six times as that of the biofuel cell employing the wild-type.

Keywords: Enzyme fuel cell; PQQ glucose dehydrogenase; Protein engineering; Bilirubin oxidase; Stability

1. Introduction

Biofuel cell systems utilize biocatalysts, such as enzymes and microorganisms, as the electrocatalysts, instead of transition metal catalysts that are utilized in the conventional fuel cell systems. Several organic compounds can be utilized in biofuel cells, especially biomass based compounds can be used as the fuels such as sugars and several alcohols, and the higher efficient energy conversion can be achieved compared with transition metal based fuel cells, theoretically. Biofuel cell can be applied not only to the biomass conversion, but also to the alternative energy source in the future implantable devices, such as implantable glucose sensors in the artificial pancreas.

Recent trends in the development of biofuel cell is the use glucose as the fuel to be oxidized at the bioanode on which glucose oxidase (GOD) is immobilized, combining with appropriate biocathode, employing several dioxygen reducing enzymes (Chen et al., 2001; Katz et al., 1999; Kim et al., 2003; Mano et al., 2002, 2003). Together with the recent advances in microfabrication technology, miniaturized biofuel cells have also been reported (Chen et al., 2001; Mano et al., 2002, 2003; Soukharev et al., 2004). GOD is an extremely stable enzyme with high substrate specificity and relatively high K_m value. Besides, in the field of self-blood glucose monitoring, glucose dehydrogenase harboring pyrroloquinoline quinone as the prosthetic group (PQQGDH) is focused owing to its oxygen insensitivity and high catalytic efficiency (Kost et al., 1998; Tang et al., 2001). Additionally its wide substrate specificity is advantageous for application of this enzyme to bioanode especially for biomass conversion. However, the application of PQQGDH to the bioanode is inherently limited because of its instability. The authors have been engaged in protein engineering of PQQGDH in order to develop the ideal enzyme

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for glucose monitoring (Sode et al., 2000; Takahashi et al., 2000).

In those studies, one engineered enzyme, the Ser415Cys mutant (S415C) was obtained, of which stability was greatly improved by protein engineering (Igarashi and Sode, 2003). The S415C is stable even at 70 °C. This is the most stable cofactor-binding GDH so far reported. The alteration of the residue at position 415 did not affect the catalytic efficiency of this enzyme, retaining high catalytic efficiency in glucose oxidation. The engineered PQQGDH therefore appears to be the most suitable enzyme for glucose based biofuel cell anode.

In this paper, we evaluated the potential application of the engineered PQQGDH, S415C, as the bioanode enzyme in glucose utilizing biofuel cell systems. We constructed an enzyme glucose biofuel cell system employing S415C in the anodic reaction and bilirubin oxidase (BOD) for the cathodic reaction. It is reported that electrochemical reduction of dioxygen to water precedes very effectively at 0.4 V versus Ag/AgCl in pH 7.0 solution at an ambient temperature mediated by the 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulfonate) (ABTS2-)-mediated and catalyzed by bilirubin oxidase (Tsujimura et al., 2001). We then investigated the stability of the bioanode to evaluate the future potential application of the protein engineered enzymes to the biofuel cell system.

2. Materials and methods

2.1. Chemicals

Calcium chloride, 3-(*N*-morpholino) propanesulfanic acid (MOPS), 1-methoxyphenazine methosulphate (m-PMS), 2,2′-azino-*bis*(3-ethylbenzothiazolone-6-sulfonic acid) diammonium salt (ABTS Diammonium Salt) and 25% glutaraldehyde were purchased from Wako Chemicals (Osaka, Japan). Pyrroloquinoline quinone (PQQ) was from Mitsubishi Gas Chemical Company, Inc. (Tokyo, Japan). Phenazine methosulfate (PMS), 2,6-dichrolophenolindophenol (DCIP), β-D-glucose, lactose, galactose, maltose was from Kanto Chemical (Tokyo, Japan). Cellobiose was from SIGMA (St. Louis, US), xylose was from Kishida Chemical Co., Ltd. (Osaka, Japan). Bilirubin oxidase was donated from Amano Enzyme (Aichi, Japan). DC protein assay kit was from Bio Rad Laboratories (California, USA). Carbon paste (0.5 g graphite powder mixed with 0.3 ml paraffin liquid) was from BSA Inc. (West Lafayette, USA). The other reagents were of analytical grade. Ultrafree MC was purchased from Millipore Co., Ltd. (Missouri, USA).

2.2. Enzyme preparation and assay

PQQGDH and S415C was prepared as described previously (Igarashi and Sode, 2003). PQQGDH activity was measured using 0.6 mM phenazine methosulphate (PMS) and 0.06 mM 2,6-dichlorophenolindophenol (DCIP) following 30 min pre-incubation in 10 mM MOPS buffer (pH 7.0) containing 1 μM pyrroloquinoline quinone (PQQ) and 1 mM $CaCl_2$ at room temperature (25 °C). The enzyme activity was determined by measuring the decrease in absorbance of dichlorophenolindophenol (DCIP) at 600 nm.

2.3. Preparation of PQQGDH and bilirubin oxidase electrode

An amout of 10 mmol MOPS buffer (pH 7.0) containing 300 U of PQQGDH or 100 U S415C or 5 U of Bilirubin oxidase was mixed with carbon paste (0.5 g graphite powder mixed with 0.3 ml paraffin liquid) and mixture was then lyophilized. The lyophilized mixture was then packed into the end of a carbon electrode (3 mm in diameter, BAS Inc., West Lafayette, USA) and treated with 1% glutaraldehyde solution for 30 min and then washed with 10 mM Tris–HCl buffer (pH 7.0). The PQQGDH or S415C immobilized electrode was then allowed to undergo holo-formation in 10 mM MOPS buffer (pH 7.0) containing 5 μM PQQ and 1 mM $CaCl_2$ at 4 °C for at least 30 min and washed with 10 mM MOPS (pH 7.0). BOD electrode was washed with 10 mM MOPS buffer (pH 7.0). All electrodes were stored at 4 °C until use.

2.4. Electrochemical measurements

An Ag/AgCl electrode (Model RE-1, BAS Inc.) and a Pt wire were used as reference and counter electrodes, respectively. The enzyme electrode (3 mm diameter, BAS Inc.) reference electrode, and counter electrode were joined into a 10 ml water-jacket cell (BAS Inc., Model VC-2) through holes in its Teflon cover. The potential was controlled by a potentiostat HA151 (Hokuto-Denko, Tokyo, Japan) in a three-electrode cell and currents were recorded with a chart recorder (Ohkura electric company, Tokyo, Japan). All measurements were carried out at 25 °C in 10 ml of the buffer with magnetic stirring (250 rpm). PQQGDH electrode was immersed in 10 mM MOPS buffer containing 1 mM m-PMS, 1 mM $CaCl_2$ and 20 mM glucose and BOD electrode was immersed in 10 mM MOPS buffer containing 0.5 mM ABTS. The physiologically relevant pH 7 was chosen for both systems. The applied potential for the calibration curve measurements of PQQGDH electrodes was +100 mV versus Ag/AgCl. The calibration curve measurements were carried out with consecutive 100 μl injections of glucose solution into the reaction cell. Cyclic voltamogram (CV) measurements of current response were carried out using the same electrode and solution for S415C electrode and BOD electrode. The potential was cycled between −400 and +200 mV for S415C electrode and between +200 and +700 mV for BOD electrode with a sweep rate of 10 mV/s using HZ3000 electrochemical analyzer (Hokuto-Denko, Tokyo, Japan).