High Current Density Bioelectrolysis of D-Fructose at Fructose Dehydrogenase-adsorbed and Ketjen Black-modified Electrodes without a Mediator

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D-Fructose dehydrogenase (FDH) has been irreversibly adsorbed without loss of the enzymatic activity on Ketjen black (KB)-modified glassy carbon electrodes, although the adsorption rate is very slow probably owing to the microstructure of KB. The FDH-adsorbed electrode produced the enzyme-kinetic-controlled catalytic oxidation wave of D-fructose at current densities of as high as 10 mA cm^{-2} without a mediator, in which the electron in FDH seems to be directly transferred to the electrode via the heme *c* site. No hindrance was observed in the mass transfer of D-fructose to KB-modified electrodes.

Escalating attention in direct electron-transfer (DET)-type bioelectrocatalysis[1] is driven by its important applications to biosensors, biofuel cells and bioreactors and by great desire to construct miniaturized and simplified bioelectrochemical devices.[1,2] DET reactions occur only at some limited kinds of electrodes suitable for individual redox enzymes (and proteins). There must be several factors governing the DET reaction of redox enzymes, but the factors have not yet clearly elucidated. In addition, the current density is usually in the order of several ten μA cm^{-2} or far below in DET-type bioelectrocatalysis. Therefore, it is strongly demanded to find new compatible couples between enzymes and electrodes and to increase the current density of DET-type bioelectrocatalysis. Improvements in enzyme immobilization methods and protein engineering have also been studied.[3]

D-Fructose dehydrogenase (FDH; EC 1.1.99.11) from *Gluconobacter* sp. is a membrane-bound enzyme with a molecular mass of ca. 140 kDa containing one flavin and one heme *c* as prosthetic groups[4] and looks promising as a catalyst of DET-type bioelectrocatalytic oxidation of D-fructose to 2-keto-D-fructose.[5] This enzyme shows high substrate specificity to D-fructose and is expected to be utilized in the field of food analysis[6] and clinical use.[7]

In this study, we attempt to develop FDH-immobilized electrodes for DET-type bioelectrocatalytic oxidation of D-fructose at current densities of as high as the order of 10 mA cm^{-2}. One of the strategies to increase the current density might be the utilization of electrodes with large roughness factor. However, the microstructure of electrodes and the immobilization of FDH should not disturb the mass transfer of the substrate in bioelectrocatalysis. Considering these factors we focused our attention to Ketjen black (KB, EC 300J) as an enzyme-adsorbing electroconductive material.

FDH was purchased from Toyobo Enzymes and used without further purifications. The commercial sample contains a surfactant. The concentration of the FDH stock solution was determined by the Lowry method with bovine serum albumin as a standard. Poly(vinylidene difluoride) (PVDF, Aldrich) was used as a binder and dissolved in *N*-methyl-2-pyrrolidone (NMP, Wako) as a 10% (w/w) solution. KB powder was ground with an agate triturator and then mixed with the PVDF solution (80:20, w/w) to prepare KB slurry. The slurry was applied to the surface of glassy carbon electrodes (GCE, 3 mm in diameter, BAS) and dried in a drying oven at 60 °C for 12 h. The KB-modified GCE, the Ag|AgCl|sat. KCl electrode, and a Pt wire were used for the working, reference, and counter electrodes, respectively. All potentials are referred to the Ag|AgCl reference electrode in this paper. Electrochemical measurements were performed using a BAS-CV 50 W electrochemical analyzer in a McIlvaine buffer of pH 5 at the room temperature (25 ± 2 °C) under anaerobic conditions.

The KB-modified GCE is completely silent toward D-fructose in the absence of FDH. On the addition of FDH (1.4 μM), a clear oxidation wave appeared, although the current density was very small in the beginning (ca. 0.4 mA cm^{-2}). Careful experiments led us to find that the oxidation current density increased very slowly with time under stirring and reached the maximum value in the range of 7–10 mA cm^{-2} after about 6 h (see Figure 1 as an example). The voltammogram is a typical catalytic wave with sigmoidal and steady-state characteristics (Figure 2, curve (B)).

Similar catalytic waves were observed at bare GCEs, but the current density was far smaller (ca. 0.1 mA cm^{-2}) than that obtained at KB-modified GCEs. QCM measurements using KB-modified gold electrodes evidenced time-dependent increases in the amount of FDH on the electrode surface (data not shown), as has been reported for bilirubin oxidase which catalyzes DET-type bioelectrocatalytic reduction of dioxygen to water.[8] Under quiet solutions, it took longer time (ca. 1 day) to attain the maximum value. In addition, almost identical cyclic voltammo-

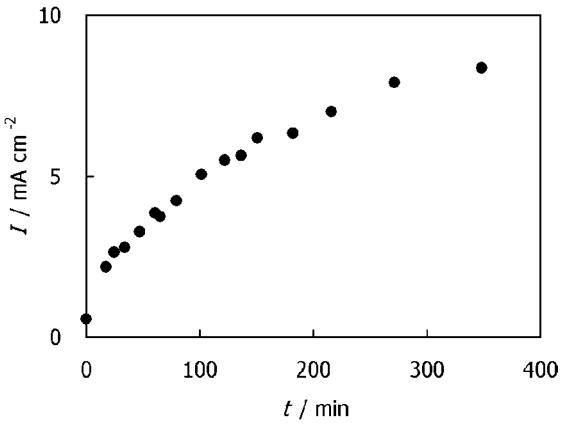

Figure 1. Time-dependence of FDH-catalyzed oxidation current density (*I*) measured at 0.5 V under stirring.

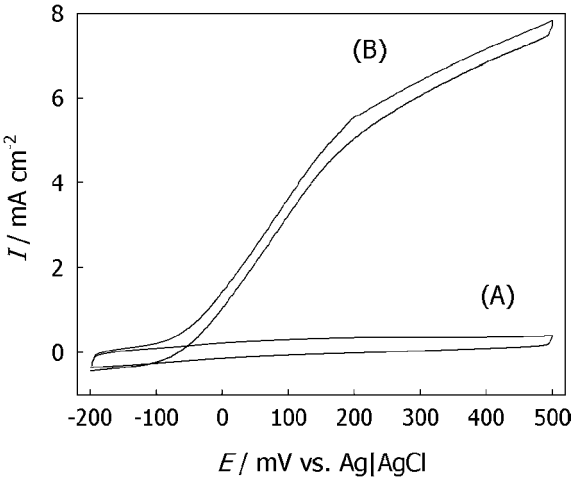

Figure 2. Cyclic voltammograms of an FDH-adsorbed and Ketjen black-modified GCE in pH 5.0 McIlvaine buffer containing 200 mM fructose in the absence (A) and the presence (B) of 200 mM fructose. The data were taken at $v = 20\,\mathrm{mV\,s^{-1}}$ about 12 h after the addition of 1.4 μM FDH.

grams were obtained when the KB-modified GCEs used were transferred to a fresh solution of D-fructose in the absence of FDH. Therefore, we can safely conclude that FDH adsorbs irreversibly on KB-modified GCE and works as a catalyst of DET-type bioelectrocatalytic oxidation of D-fructose. As far as we know, this is the first report on DET-type bioelectrocatalytic oxidation of substrates at such high current density.

Figure 1 indicates a slow kinetics in the adsorption of FDH on a KB-modified electrode. At bare GCEs (or pyrolytic graphite electrodes) without KB, however, the catalytic wave was not affected by time of the electrode standing. Therefore, the slow adsorption kinetics seems to be ascribed to the adsorption of FDH to some microstructural regions of KB.

After reaching the maximum value, the steady-state voltammograms such as curve (B) in Figure 2 were not affected by stirring. This means that the steady-state current was controlled by the enzyme kinetics and that there occurred no concentration polarization of D-fructose at least at 200 mM. In contrast with FDH, D-fructose seems to be supplied easily to the microstructural regions of KB, probably because of small molecular size of fructose. All these factors seem to be responsible for high current density of the DET-type bioelectrocatalytic oxidation of D-fructose.

The anodic current began to increase around 0 mV (Figure 2, curve (B)). The value is close to the redox potentials of FDH observed by differential pulse voltammetry in the absence of fructose at pH 4.5 (80, 80, and 40 mV for Pt, Au, and GCE, respectively).[5b] Although the redox potential was not assigned in the literature, the values would be assigned to the heme *c* group since the redox potential of flavin is more negative than the values. Therefore, it can be concluded that the flavin site in FDH accepts electrons from D-fructose and transfers the electrons to the heme *c* site. The heme *c* site donates the electrons to the carbon electrodes.

The D-fructose concentration dependence of the steady-state catalytic current density (at 0.5 V) followed a Michaelis–Menten-type equation, where the current was measured under stirring.

$$I = \frac{I_{\max}}{1 + K_{\mathrm{M}}/c_{\mathrm{f}}} \quad (1)$$

where K_{M} is an apparent Michaelis constant for D-fructose including a factor representing the mass transfer of the substrate, c_{f} is the bulk concentration of D-fructose, and $I_{\max}$ is the maximum current density. The $I_{\max}$ values varied from 1 to 10 mA $\mathrm{cm^{-2}}$ depending on the amount of KB on GCEs, while the K_{M} value located in the range of 9–10 mM. The K_{M} values are close to that obtained in the case of enzymatic reaction in bulk solution (10 mM).[4] The result supports the idea that the concentration depression of the substrate is negligible even near the microstructural electrode surface. This feature is different from that observed for enzyme multilayer systems.[9] Therefore, most probably, FDH adsorbs as a monolayer on KB-modified electrodes.

The present study has shown that FDH adsorbed on KB-modified electrodes exhibits the high catalytic activity of the electrocatalytic oxidation of D-fructose without a mediator. The electrodes may be utilized for the third generation biosensors (in coulometric as well as amperometric detection), biofuel cell anodes, and also bioreactors.

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