Investigation of a Tyrosinase-Immobilized Polyacrylamide Membrane Electrode in *N*,*N*-Dimethylacetamide

Jujie Ren, Hiroyuki Takeda,
and Toshio Nakamura*

Faculty of Science, Shinshu University,
3-1-1 Asahi, Matsumoto 390-8621

Received March 15, 2006; E-mail: toshion@shinshu-u.ac.jp

A tyrosinase-immobilized, hydrophilic polyacrylamide membrane electrode was used to investigate the enzymatic reaction of catechol derivatives in DMA and the apparent Michaelis–Menten constants, $K_{\mathrm{m,app}}$, were determined. The method can be used for both monitoring enzymatic activity and solvent effect using only a small amount of enzyme.

Many studies exploiting enzymatic activity in nonaqueous solutions have been carried out after Dastoli and Price reported their investigation of the catalytic activities of xanthine oxidase suspended in several organic media.[1] Enzymes have been found to have numerous applications as practical and specific catalysts in chemical and pharmaceutical syntheses and as recognition elements in biosensors. The discovery that they are catalytically active in organic solvents containing little or no water has expanded their usefulness further still.[2–4] Some of the benefits in utilizing enzymes in nonaqueous solvents over the use of conventional aqueous reaction media include the high solubility of many hydrophobic substrates in such solvents, the suppression of various side reactions promoted by water, altered enzymatic selectivity, and the ability to control it with the solvent. Many studies have been conducted in order to gain an understanding of the so-called solvent effect due to organic solvent properties. Factors such as solvent hydrophobicity,[5] solvent polarity, water content,[6] and substrate hydrophobicity[7] can affect the enzymatic reaction rate. Still, it is worthwhile to find new enzymatic reaction systems for research and practical use, especially in dipolar aprotic solvent.

Recently, there is an increasing interest in studies of enzyme electrodes in various organic solvents.[8–16] Dipolar aprotic solvents are important reaction media and have been widely used in place of water in experimental laboratories, industry, and many other domains. There are obvious incentives for developing enzyme electrodes and investigating the properties of this type of biosensor for use in dipolar aprotic solvents. An electrode system that is made by immobilizing an enzyme in a hydrophilic thin polymer membrane attached an electrode make it possible to investigate conveniently the enzyme activity at the membrane–organic solution interface using smaller amounts of enzyme compare with batch methods.

Updike and Hicks published the results of their experiments involving an enzyme electrode with cross-linked polyacrylamide (PAA) membrane as a matrix for use in aqueous solutions.[17] Hydrophilic non-cross-linked polyacrylamide has been used in our laboratory as a matrix to construct bioelectrochemical systems by immobilizing biofunctional materials,[18] and it has been shown that the membrane electrode has array-like properties.[19] The kinetic properties of a tyrosinase-immobilized PAA electrode with some substrates of catechol derivatives in acetonitrile (AN) have been studied.[20]

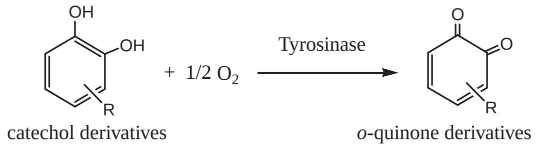
To verify the catalytic properties of tyrosinase in a new environment, PAA membrane was immersed in a dipolar aprotic solvent with a low water content ($<0.01\%$ w/w), and the effect of organic solvents on the enzymatic reaction and the properties of the electrode in *N*,*N*-dimethylacetamide (DMA) were studied. Some possible kinetic factors related to the enzymatic reactions occurring in new this environment are proposed in this paper, and solvent effects due to dipolar aprotic solvents for these reactions are also described.

Cyclic voltammetric measurements of the PAA–tyrosinase electrode were carried out for the substrates of 4-methylcatechol, catechol, and biphenyl-2,3-diol in a 50 mM $\mathrm{Et_4NClO_4}$ *N*,*N*-dimethylacetamide (DMA) solution. In the absence of the substrates, both the Pt electrode and the PAA–tyrosinase electrode gave no detectable signal. When the substrates were added no response was observed for the Pt electrode, while reduction peaks occurred for each substrate at the PAA–tyrosinase electrode, which was attributed to the reduction of the enzymatic reaction product, *o*-quinone. It was produced through the enzymatic oxidation of *o*-dihydroxybenzene with dissolved oxygen at the boundary of the organic solvent and PAA membrane, then diffused to the surface of the Pt across the membrane, and was reduced by the electrode (Scheme 1).

Typical cyclic voltammograms of 0.5 mM 4-methylcatechol in DMA solution are shown in Fig. 1. The voltammogram using the PAA–tyrosinase electrode showed a reduction peak at -0.93 V, which is more negative than that in AN, as in Table 1. Basically, the reduction state of quinone in the electrochemical reaction is solvated more by AN than by DMA because DMA is more acidic (the acceptor number of DMA, 13.6, is lower than that of AN, 18.9[21]). The reduction peak potentials of the enzymatic reaction product of substrates on the PAA–tyrosinase electrode in 50 mM $\mathrm{Et_4NClO_4}$–DMA and AN solution are shown in Table 1.

A typical current–time plot for the PAA–tyrosinase electrode upon stepwise addition of 4-methylcatechol at -0.90 V is shown in Fig. 2. When the substrate was added into Cell 1, the reduction current rose steeply to reach a stable value. The applied potential was set at -0.900 V in the experiment because the reduction of O_2 occurs at a more negative potential.

Figure 3 shows the calibration plot of the PAA–tyrosinase electrode for 4-methylcatechol in 50 mM $\mathrm{Et_4NClO_4}$–DMA



Scheme 1.

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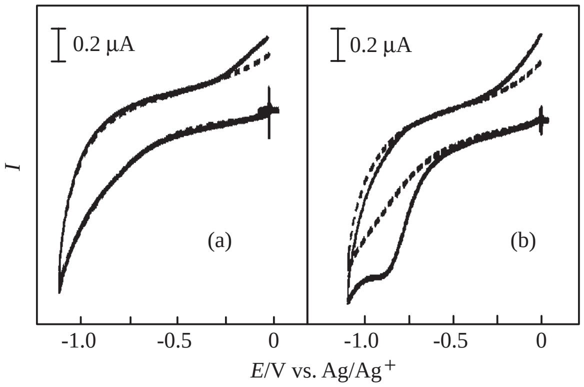

Fig. 1. Cyclic voltammograms of 4-methylcatechol in 50 mM Et_4NClO_4–DMA solution at (a) the Pt and (b) the PAA–tyrosinase electrode. Dotted and solid lines are for blank and sample solutions, respectively. Scan rate is $0.100\,V\,s^{-1}$.

Table 1. Reduction Peak Potentials of the Enzymatic Reaction Product of Substrates on the PAA–Tyrosinase Electrode in 50 mM Et_4NClO_4–DMA and AN Solution at 35 °C

Substrate	Peak potential/V	
	in DMA	in AN[20]
4-Methylcatechol	−0.93	−0.70
Catechol	−0.98	−0.65
Biphenyl-2,3-diol	−1.06	−0.67

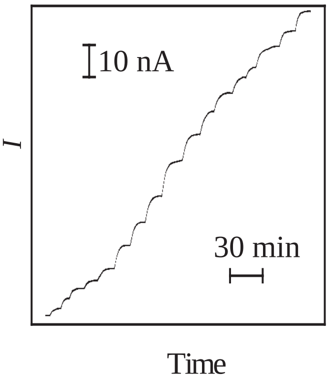

Fig. 2. Typical current–time response curve of the PAA–tyrosinase electrode upon stepwise additions of 4-methylcatechol from 0.35 to 35 mM in 50 mM Et_4NClO_4–DMA solution at −0.900 V.

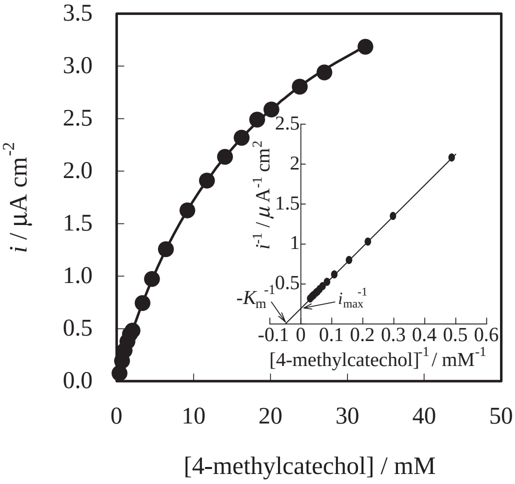

Fig. 3. Calibration plot of the PAA–tyrosinase electrode for 4-methylcatechol in 50 mM Et_4NClO_4–DMA solution at −0.900 V. Inset: Lineweaver–Burk plot for the response of the PAA–tyrosinase electrode to 4-methylcatechol in DMA.

solution at −0.900 V. The response of the electrode is characteristic of the Michaelis–Menten kinetic mechanism. The apparent Michaelis–Menten constant, $K_{m,app}$, of tyrosinase in PAA-electrode to substrate was calculated according to the Lineweaver–Burk equation,[22–24] $i^{-1} = i_{max}{}^{-1}K_{m,app}[S]^{-1} + i_{max}{}^{-1}$, where [S] is the concentration of substrate.

The inset of Fig. 3 shows the Lineweaver–Burk plot for the response of the PAA–tyrosinase electrode to 4-methylcatechol in DMA. The apparent constant $K_{m,app}$ for the reaction of tyrosinase with 4-methylcatechol at the electrode was calculated to be 20 mM. The $K_{m,app}$ for the tyrosinase at the PAA-electrode with biphenyl-2,3-diol and catechol were also calculated to be 5 and 2 mM, respectively. The $K_{m,app}$ value, which is related to the enzymatic affinity, of tyrosinase-immobilized in PAA toward catechol is similar to that for the enzyme in aqueous solution.[25] Therefore, tyrosinase remains active towards substrates dissolved in a dipolar aprotic solvent, DMA, although the enzyme exists in a PAA matrix.

Enzymes are considered to be active in aqueous systems. When an enzyme is placed in an organic solution and still remains active, the enzyme activity is related to the amount of water bound to the enzyme.[5,26,27] The water bound to the enzyme in an organic solvent may not form a layer covering the enzyme. It may be just a few clusters around charged and polar regions on the enzyme surface, which can be regarded as the regions where the enzymatic reaction occurs. So, even in an organic solvent with a low water content, an enzyme still exhibits some activity.[27] In this study, the tyrosinase remains active because tyrosinase in the hydrophilic PAA membrane, which behaves as a so-called membrane protein,[28] is naturally accustomed to a nonaqueous environment.

As well, the tyrosinase-immobilized, hydrophilic PAA membrane-coated platinum electrode exhibits characteristics of the Michaelis–Menten kinetic mechanism towards the substrates mentioned above in a dipolar aprotic solvent, DMA, even when the water content is lower than 0.01%. The apparent Michaelis–Menten constant $K_{m,app}$ of tyrosinase in PAA for catechol is similar to that for the enzyme in aqueous solution. The electrode used in the paper could be used to monitor solvent effects on enzyme activities and their reaction kinetics.

Experimental

Reagent grade DMA was purchased from Wako and distilled twice under reduced pressure (ca. 15 mmHg) and nitrogen after removing water with molecular sieves. The first distillation was carried out in the presence of BaO, and the second in the presence of CaH_2. The water content of the solvents measured by Karl Fischer titration was lower than 0.01%. Tyrosinase was purchased from Sigma-Aldrich Co. and stored at 4 °C in the dark. Ferrocene

(Fc) and catechol were purchased from Tokyo Kasei Kogyo Co., Ltd., and 4-methylcatechol and biphenyl-2,3-diol were purchased from Wako Pure Chemical Industries Ltd. Polarographic grade tetraethylammonium perchlorate (Et_4NClO_4) was purchased from Nacalai Tesque. The polyacrylamide was a product of Aldrich, Inc. (No. 18127-7).

The tyrosinase-immobilized polyacrylamide membrane electrode (PAA–tyrosinase electrode) was constructed by placing 20 µL of an aqueous solution containing 0.4 mg PAA and 180 units tyrosinase on the end of a platinum rod with a diameter of 2 mm housed in a Teflon body and then by keeping the electrode in a silica-gel desiccator for 2 h after blowing the drop almost dry with nitrogen. The thickness of the membrane on the platinum disk was less than 0.02 mm. The PAA electrode was the same as the PAA–tyrosinase electrode except for the absence of the enzyme. A schematic diagram of the electrode was included in a previous report.[29] These electrodes were conditioned for one hour in 50 mM Et_4NClO_4–DMA solution before use.

Cyclic voltammetric and amperometric measurements of Cell 1 were carried out at 35 °C using an arbitrary function generator (HB105 Hokuto Denko HB105) and a potentiostat/galvanostat (HA150). Voltammograms were recorded with an X–Y recorder (Rikadenki Industry Co., Ltd., Model RW-11). The electrochemical cell was assembled using a three-electrode system: a working electrode (PAA or PAA–tyrosinase electrode), an Ag^+/Ag reference electrode, and a Pt counter electrode. The solutions were stirred with a stirrer at a stated speed when amperometric experiments were carried out. The solutions were stirred for four minutes and then allowed to stand for one minute before cyclic voltammetric experiments were performed.

$$\begin{aligned}&\mathrm{Ag} \mid 10\,\mathrm{mM}\ \mathrm{AgNO_3} + 10\,\mathrm{mM}\ \mathrm{Et_4NClO_4(AN)} \parallel \\ &50\,\mathrm{mM}\ \mathrm{Et_4NClO_4}\ \mathrm{(DMA)} \parallel \mathrm{S} + 50\,\mathrm{mM}\ \mathrm{Et_4NClO_4}\ \mathrm{(DMA)} \mid \\ &\mathrm{PAA\text{–}tyrosinase} \mid \mathrm{Pt} \cdots \mathrm{Cell\ 1}\end{aligned} \tag{1}$$

In Cell 1, S denotes 4-methylcatechol, catechol, and biphenyl-2,3-diol. The change in liquid-junction potentials of Cell 1 during emf measurements was considered negligible.[30,31]

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