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Electrochemical Behavior of Catechol and 3,4-Dihydroxytoluene in Acetonitrile at a Platinum-Disk Electrode Modified with a Tyrosinase Containing Polyacrylamide Film

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A modified platinum-disk electrode coated with a non-plasticized polyacrylamide (PAA) membrane was used to study electrochemically an enzymatic reaction between tyrosinase in the PAA membrane and catechol and 3,4-dihydroxytoluene in acetonitrile (AN). Tyrosinase, a hydrophilic biofunctional material, was immobilized in the thin PAA membrane, which adhered to the platinum-disk electrode and was stable in AN. The enzymatic activity of tyrosinase in the PAA membrane to the above substrates in AN was confirmed by cyclic voltammetry and amperometry. The apparent maximum velocities (V_{max}^{app}) and the apparent Michaelis constants (K_m^{app}) were determined from the amperometric results; the apparent turnover numbers were also determined. The reduction potentials of the substrates were reported *vs*. the cathodic peak potential of ferrocene in AN to improve the reliability of the potential data and to make possible a comparison of the potentials in different solvents. The electrochemical system discussed in this report can be used for tracing enzymatic reactions with substrates dissolving in aprotic dipolar solvents and for investigating solvent effects on enzymatic activities.

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Introduction

A number of studies on the use of enzymatic activity in nonaqueous solutions have been carried out after Dastoli and Price reported about catalysis by xanthine oxidase suspended in several organic media.[1] In this work they found that several kinds of solvents offered to enzymes a field to show catalytic activities; however, the maximal velocities were less by factors of tenfold compared with those obtained for the enzyme-catalyzed reaction in aqueous solution. An enzyme electrode for use in aqueous solutions was reported by Updike and Hicks by coating a platinum disk coated with a cross-linked polyacrylamide (PAA) membrane containing glucose oxidase.[2] Whitesides *et al*. also used cross-linked PAA gels to immobilize enzymes in aqueous solutions.[3] A tyrosinase was used in chloroform,[4] in low-water content organic solvents,[5] and in such nonaqueous solvents as chloroform, chlorobenzene, and 1,2-dichlorobenzene.[6] In Ref. 6, Deng and Dong reported an amperometric response of a tyrosinase-immobilized Nafion membrane electrode which was inhibited by some inhibitors. We have found from the results obtained in our previous research[7] that an ionophores-conjugated PAA-coated platinum disk electrode could be used for obtaining voltammetric information on cations and anions in AN.[8,9] It will be worth while to develop new types of enzyme electrodes for use in dipolar aprotic solvent systems, in which we can dissolve many hydrophobic substrates, extend the potential windows, trap intermediate reaction products like radicals, and, as a result, trace new enzymatic activities and the reaction mechanism. Thus, we may expect to obtain new sensing systems. Furthermore, it is important to obtain the kinetic parameters of the enzymatic reaction which proceeds in dipolar aprotic solvents. We reported that platinum-disk electrodes modified with a thin PAA membrane, which was stable in nonaqueous solutions, could immobilize hydrophilic biofunctional materials in a thin PAA membrane tightly adhered to a platinum disk.[7] Tyrosinase is generally known as an enzyme which catalyzes the oxidation of the phenol and catechol derivatives, and is also related to the formation of melanin pigment. It also holds its enzymatic activity in nonaqueous solvents, as mentioned above. Here, we report on the enzymatic and electrochemical behavior of a modified platinum disk electrode coated with non-plasticized PAA containing tyrosinase for catechol and 3,4-dihydroxytoluene in AN. Furthermore, we used ferrocene (Fc) as the pilot substance to standardize the reduction potentials obtained in dipolar aprotic solvents.

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Experimental

Reagents

Tyrosinase and 3,4-dihydroxytoluene were purchased from Wako Pure Chemical Industries Ltd. (No. 208-11531 and 043-02412) and the former was stored at 4˚C in the dark. Pyrogallol, *p*-cresol, and tetraethylammonium perchlorate (Et_4NClO_4) were purchased from Nacalai Tesque (No. 297-03, 097-08, and 330-16). Acetonitrile (Wako No. 041-00386) was purified two times by fractional distillation after removing water with molecular sieves from Nacalai Tesque (No. 233-11), first from phosphorous pentaoxide and second from calcium hydride. Catechol and ferrocene were purchased from Tokyokasei Kogyo Co. Ltd. (No. P0567 and D0444). Polyacrylamide was a product of Aldrich, Inc. (No. 18127-7).

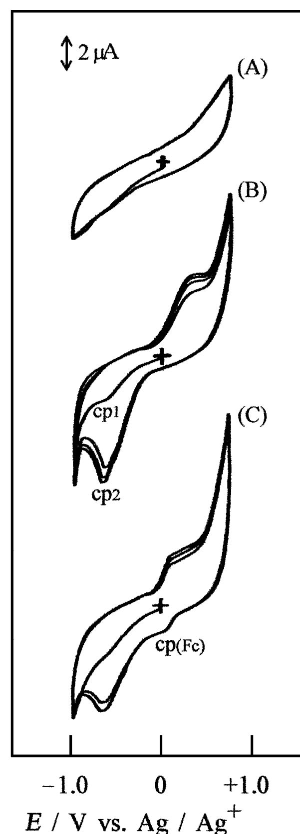


Fig. 1 Cyclic voltammograms of catechol (A) 0 mM, (B) 1.5 mM, and (C) 1.5 mM + 0.1 mM Fc in 50 mM Et_4NClO_4–AN recorded at the PAA-tyrosinase electrode after standing for 5 min at the rest potential. cp1 and cp2, peak currents obtained at the first and third scans, respectively; cp (Fc), cathodic peak of Fc; scan rate, 0.100 V s^{-1}.

Indicator electrode

The PAA-tyrosinase electrode was constructed by applying a platinum disk (2 mmϕ) 20 μL aqueous solution containing 0.4 mg PAA and varying the amounts of tyrosinase and by keeping it in a silica-gel desiccator for 2 h. The thickness of the membrane on the platinum disk seemed to be less than 0.3 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 construction was shown in a previous report.[7,10] These electrodes were conditioned for at least 2 h in a 50 mM Et_4NClO_4–AN solution.

Electrochemical measurement

The cyclic voltammetric (CV) and amperometric measurements of Cell 1 (described below) were carried out at 35˚C with both a potentiostat/galvanostat (Hokuto Denko Co., Ltd., Model HA150) and an arbitrary function generator (Hokuto Denko Co., Ltd., Model HB105). Voltammograms were recorded with an X-Y recorder (Rikadenki Industry Co., Ltd., Model RW-11). An auxiliary electrode was a platinum wire.

Ag|10 mM $AgNO_3$ + 10 mM Et_4NClO_4(AN)||50 mM Et_4NClO_4(AN)||S + 50 mM Et_4NClO_4(AN)|PAA-tyrosinase|Pt...Cell 1

In Cell 1, S denotes catechol, 3,4-dihydroxytoluene, and Fc (M = mol dm^{-3}).

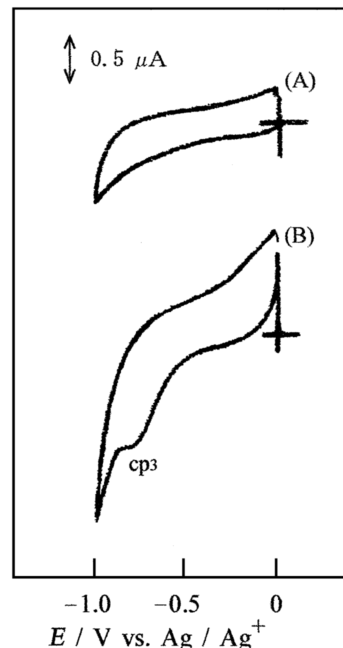
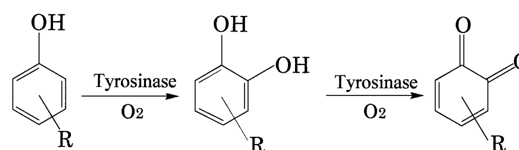


Fig. 2 Cyclic voltammograms of 3,4-dihydroxytoluene (A) 0 mM, and (B) 0.6 mM in 50 mM Et_4NClO_4–AN recorded at the PAA-tyrosinase electrode after standing for 5 min at the rest potential. Scan rate, 0.100 V s^{-1}.

Results and Discussion

CV measurement

Tyrosinase caused the following enzymatic reaction (Scheme 1) in aqueous solution:



Scheme 1

In this work CV measurements were carried out with Cell 1; voltammograms at the PAA-tyrosinase electrode for catechol and 3,4-dihydroxytoluene in AN are shown in Figs. 1 – 3. The cathodic peaks (cp) were observed at about –0.65 V *vs*. Ag/Ag^+ (cp1,2) for catechol and about –0.70 V *vs*. Ag/Ag^+ (cp3) for 3,4-dihydroxytoluene. The peaks, cp1 and cp3, were not obtained at the PAA electrode. cp1 in Fig. 3 was the same as that shown in Fig. 1; however, it was recorded at a different current scale. Cell 1 was allowed to stand at rest potential before the CV scan and peak current (cp2) was saturated within 5 min to a reproducible value. The rest potential was monitored during the standing time, and it also approached a steady value within 5 min. The peak currents, i_{cp1} and i_{cp3}, seem to have been obtained by the enzymatic reaction products produced by the oxidation of catechol and 3,4-dihydroxytoluene in AN being catalyzed by tyrosinase during 5 min standing at rest potential. However, we obtained no such cathodic current peaks (cp1 – 3), for pyrogallol and *p*-cresol at both the PAA-tyrosinase and PAA electrodes. On the other hand, i_{cp2} seems not to be obtained only by an enzymatic reaction, but also by an electrode reaction. Because

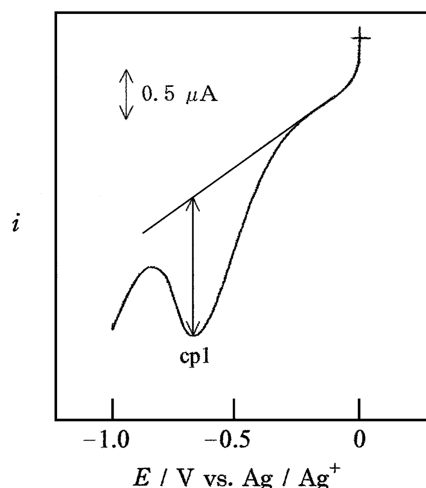


Fig. 3 Cyclic voltammogram of 1.5 mM catechol in 50 mM Et_4NClO_4–AN recorded at the PAA-tyrosinase electrode after standing for 5 min at the rest potential. Scan rate, 0.100 V s^{-1}.

Table 1 Current density and redction potential of catechol in AN obtained by repeated measurements at a PAA-tyrosinase electrode after standing for 5 min at the rest potential (35˚C)

n	$I_{cp2}/10^2$ μA cm^{-2}	E_{cp2} *vs.* $E_{cp(Fc)}$/V
1	1.33	-0.69_2
2	1.34	-0.70_0
3	1.31	-0.70_0
4	1.33	-0.71_0
5	1.35	-0.71_0
Ave.	1.33 ± 0.01	-0.70_3

The values were obtained at the third scan. [catechol] = 1.5 mM; scan rate: 0.100 V s^{-1}.

no electrolytic procedure was contained before obtaining i_{cp1} and i_{cp3}, we may conclude that only an enzymatic reaction between tyrosinase immobilized in polymer membrane and catechol derivatives in AN could proceed and be traced by voltammetry. The relations between the concentration of catechol and i_{cp2} and between that of 3,4-dihydroxytoluene and i_{cp4}, the cathodic peak current at the third scan for 3,4-dihydroxytoluene, were plotted. They were linear from 0.01 mM to 0.4 mM of catechol with a slope of 2×10^5 μA cm^{-2} M^{-1} (r^2 = 0.9812) and from 0.1 mM to 0.8 mM of 3,4-dihydroxytoluene with a slope of 2×10^4 μA cm^{-2} M^{-1} (r^2 = 0.9821), respectively, both relations crossing the origin. Repeated measurements of CV at 1.5 mM of catechol showed reproducible results, as shown in Table 1. The reduction potential of 3,4-dihydroxytoluene (E_{cp3}) *vs.* the cathodic current peak of Fc (cp(Fc)) was -0.75_5 V, about 0.050 V more negative than that of catechol. The tyrosinase concentration contained in the PAA membrane was changed, and it was found that 100 units of tyrosinase in the PAA membrane was sufficient for 3 mM catechol in AN. The potentials given in Table 1, which are referred to as the cathodic peak potential of the Fc/Fc^+ system, have advantages in that they are reliable when using different solvent systems, and can be compared with the potentials in different solvents.[11] Figure 4 shows a plot of the results obtained by CV measurement for 3,4-dihydroxytoluene in AN both at the PAA-tyrosinase and the PAA electrodes. We can consider from the difference in the peak currents, as shown in the figure, that the peak at the PAA electrode obtained after the third scan was the reduction current of an oxidized product, not of the enzymatic reaction, but of the electrolytic reaction. On the other hand, the cathodic current obtained at the PAA-tyrosinase electrode seems to consist of both the enzymatic reaction and the electrolytic reaction.

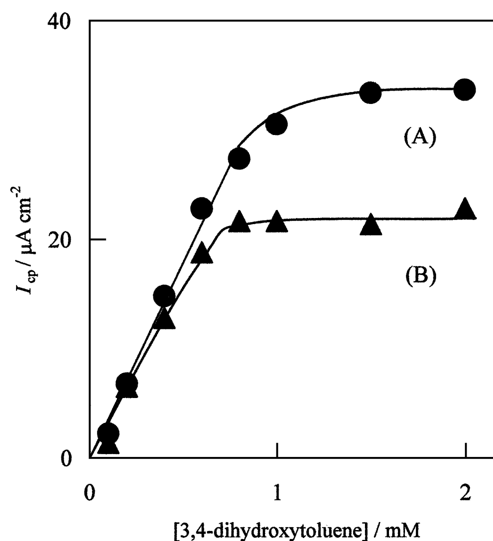


Fig. 4 Relation between the concentration of 3,4-dihydroxytoluene and the cathodic peak current density at the third scan in 50 mM Et_4NClO_4–AN after standing for 5 min at the rest potential. Scan rate, 0.100 V s^{-1}. (A) at the PAA-tyrosinase electrode. (B) at the PAA electrode.

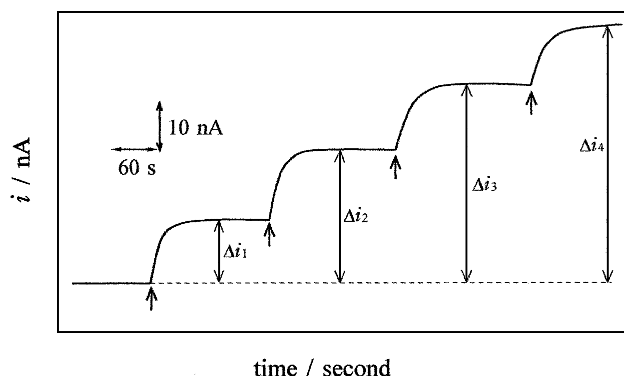


Fig. 5 Dynamic amperometric response at the PAA-tyrosinase electrode upon successive additions of 25 mM 3,4-dihydroxytoluene in a 50 mM Et_4NClO_4–AN solution. The arrows show the addition of substrates. The concentrations of the substrate were 0.1, 0.2, 0.3, and 0.4 mM, respectively. Δi, see the text; applied potential, –0.800 V.

Amperometric measurement

Based on the results obtained by CV, an amperometry at Cell 1 was carried out under stirring the test solution during the measurement. A typical current–time relation after a stepwise addition of 3,4-dihydroxytoluene to Cell 1 is shown in Fig. 5. A good linear correlation was obtained between the cathodic current density and the concentration for both catechol (0 – 1.0 mM) and 3,4-dihydroxytoluene (0 – 1.5 mM). The slope (nA cm^{-2} M^{-1}) was 0.59×10^6 (r^2 = 0.9948) for catechol and 1.3×10^6 (r^2 = 0.9944) for 3,4-dihydroxytoluene.

Table 2 Values obtained at the PAA-tyrosinase electrode for the enzymatic reaction with different substrates in AN at 35˚C

Substrate	K_m^{app}/mM	V_{max}^{app}/nA cm^{-2}	TN/s^{-1}	E_{cp} *vs.* E_{Fc}/V
Catechol	6.6 ± 0.4 (n = 3)	(4.4 ± 0.4) × 10^3 (n = 3)	1.4 ± 0.05 (n = 3)	–0.70₃ (n = 5)
3,4-Dihydroxytoluene	5.9 ± 0.3 (n = 3)	(9.3 ± 1.0) × 10^3 (n = 3)	3.9 ± 0.1 (n = 3)	–0.75₅ (n = 5)

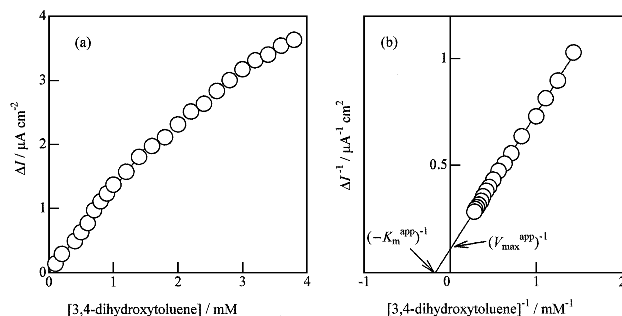


Fig. 6 (a) Effect of the concentration of 3,4-dihydroxytoluene on the reaction rate in 50 mM Et_4NClO_4–AN at the PAA-tyrosinase electrode. (b) Lineweaver–Burk reciprocal plot.

Amperometric experiments under a similar condition to that for Fig. 5 were carried out for catechol and 3,4-dihydroxytoluene in AN at the PAA electrode. Although the substrates were added stepwise, the currents did not increase. From the results, we can conclude that to obtain the amperometric current at Cell 1 the electrode needs tyrosinase in PAA, which strongly supports the occurrence of an enzymatic reaction at the PAA-tyrosinase electrode. Furthermore, we carried out an experiment under similar conditions to that for Fig. 5 in order to obtain information about the necessity of oxygen in obtaining the amperometric current at the PAA-tyrosinase electrode for both catechol and 3,4-dihydroxytoluene in AN. When the substrates were added stepwise to a solution containing dissolved oxygen, the current increased stepwise. After the last addition, deaeration with nitrogen gas was carried out. The current flow then ceased once, but as soon as oxygen was introduced into the cell again the current revived. From the results, we can conclude that the PAA-tyrosinase electrode needs oxygen to produce the amperometric current, and that it also supports the occurrence of the tyrosinase enzymatic reaction at the cell.

The reaction rate was obtained by the steady state amperometric current (Δi_i) upon each addition of the substrates, even though the value was converted into the current density, as shown in Fig. 5. The relation between the reaction rates and the concentration of the 3,4-dihydroxytoluene is plotted in Fig. 6(a). Then, from a Lineweaver–Burk reciprocal plot, like that shown in Fig. 6(b), the apparent reaction parameters, V_{max}^{app} and K_m^{app}, in the Michaelis–Menten equation were determined. The turnover numbers (TN) of the reactions for catechol and 3,4-dihydroxytoluene were also determined; the obtained values are summarized in Table 2 along with their units. Because the V_{max}^{app} for catechol was almost the same as that for 3,4-dihydroxytoluene, the substitution of a hydrogen atom of the benzene ring for a methyl group showed little effective influence on those. The differences in the cathodic peak potentials between these two substrates might be suitable for developing a sensor to distinguish derivative substrates catalyzed by the same enzyme.

In the experiment we could confirm that enzymatic reactions proceed between an enzyme in a polymer membrane and substrates in dipolar aprotic solvents, and that we could obtain the reaction parameters. The system developed here will be applicable to such substrates which dissolve neither in aqueous solvents nor in nonpolar solvents. Consequently, the system could be used to investigate solvent effects for enzymatic reactions which proceed in dipolar aprotic solvents with a quite small amount of enzyme. They should be important in obtaining information concerning organic enzymatic syntheses and to develop an enzymatic sensor for use in such solvents. A similar study concerning the reaction between cholesterol oxidase in a PAA-modified electrode and cholesterol in AN is now in progress at our laboratory.

Conclusion

Tyrosinase maintained its enzymatic activity in dipolar aprotic solvents and a platinum-disk electrode coated with the tyrosinase-containing PAA membrane could be used to trace enzymatic reactions with such substrates as catechol and 3,4-dihydroxytoluene dissolved in AN. Furthermore the electrode could be used to obtain apparent enzymatic reaction parameters proceeding in dipolar aprotic solution systems.

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