

Sensitive Simultaneous Determinations of 1,2-Dihydroxynaphthalene and Catechol by an Amperometric Biosensor

Ali A. GHOZALI,*¹ Yusuke KUMANO,*² Hirosuke TATSUMI,*^{2†} Dyah ISWANTINI,*³ Cecep KUSMANA,*⁴ and Novik NURHIDAYAT*⁵

*¹ Graduate School of Environmental and Natural Resources Management Sciences, IPB University, Jl. Raya Dramaga, Bogor 16680, Indonesia

*² Department of Chemistry, Faculty of Science, Shinshu University, 3-1-1 Asahi, Matsumoto, Nagano 390-8621, Japan

*³ Department of Chemistry, Faculty of Mathematics and Natural Sciences, IPB University, Jl. Raya Dramaga, Bogor 16680, Indonesia

*⁴ Department of Silviculture, Faculty of Forestry, IPB University, Jl. Raya Dramaga, Bogor 16680, Indonesia

*⁵ Central Research Department of Biology, Indonesian Institute of Sciences (LIPI), Jl. Raya Bogor Km. 46, Cibinong, Bogor 16911, Indonesia

An amperometric biosensor for 1,2-dihydroxynaphthalene (DHN) and catechol (Cat) has been developed in order to monitor the biodegradation of polycyclic aromatic hydrocarbons (PAHs). DHN is a common intermediary metabolite in naphthalene and phenanthrene degradation, while Cat is produced by further degradation. These compounds were detected by a biosensor modified with pyrroloquinoline quinone-dependent glucose dehydrogenase (PQQ-GDH). The biosensor was based on signal amplification by enzyme-catalyzed redox cycling and was able to detect DHN and Cat at very low concentrations down to 10^{-9} M. Since the anodic waves of DHN and Cat were well separated, simultaneous determinations of these compounds were possible. Although the current signal for DHN was reduced in repeated measurements due to the oxidative polymerization of DHN, it can be avoided when the concentration of DHN was sufficiently low ($<1 \mu\text{M}$).

Keywords Biosensors, polycyclic aromatic hydrocarbons, enzyme electrodes, redox cycling, signal amplification

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Introduction

Mangrove forests have unique ecosystems with rich biodiversity in intertidal zones. The ecosystem is formed in flat coastal areas, which are readily available for human beings, and thus more likely to be affected by human activities than other forest ecosystems. In mangrove forests, polycyclic aromatic hydrocarbons (PAHs) are regarded as being one of the most serious pollutants, because of their persistent nature.^{1,2} One possible approach to remove these pollutants is bioremediation, in which PAHs are degraded by microorganisms. Many reports have appeared concerning PAH-degrading bacteria isolated from mangrove sediments.³⁻⁶

In such bioremediation studies, it is necessary to estimate the PAH-degrading activity of the microorganisms. For this purpose, large and expensive instruments (high-performance liquid chromatography,⁷ gas chromatography,⁸ gas chromatography-mass spectrometry,⁹ etc.) have been used so far. Those instruments can give accurate analytical results, but they are often costly, and sometimes require troublesome and

time-consuming pretreatments. For the rapid screening of PAH-degrading microorganisms, a simple, fast, and inexpensive analytical method has been desired.

Electrochemical analysis is one of the most promising alternatives, because it has these desired characteristics. It also has an advantage of being free from the color or turbidity of the test solution, and thus can be applied directly to cell suspensions, for example, a liquid culture medium of the PAH-degrading microorganism. Unfortunately, PAHs are very stable and do not give any signal in usual electrochemical measurements. Reshetilov *et al.*^{10,11} have proposed a biosensor for PAHs, which consists of a Clark oxygen electrode and PAH-degrading bacteria immobilized on the electrode. The amperometric signal for dissolved oxygen is varied according to the oxygen consumption by the bacteria in the presence of PAHs. However, it is not specific to PAHs and subject to interference from many other compounds. Another approach is to detect the metabolites of PAHs. It is known that naphthalene and phenanthrene are metabolized to 1,2-dihydroxynaphthalene (DHN) by the dioxygenase enzyme.^{12,13} By further degradation, catechol (Cat) is also formed.¹² It is possible to develop a new method for evaluating the PAH-degrading ability, based on the detection of these electroactive metabolites.

One feature required for an analysis of this purpose is the

† To whom correspondence should be addressed.
E-mail: tatsumi@shinshu-u.ac.jp

sensitivity. The solubility of PAHs in aqueous media is very low, even in the case of the most soluble naphthalene. Therefore, the measurement of DHN and Cat must be performed at very low concentrations. In our recent study, enzyme-catalyzed signal amplification was introduced to the amperometric detection of the hydroxyl radical.^{14,15} In the presence of an aromatic trapping agent, pyrroloquinoline quinone-dependent glucose dehydrogenase (PQQ-GDH, E.C. 1.1.5.2) as a catalyst, and its substrate D-glucose, the current signal for the trapping adducts from hydroxyl radical was amplified and detected sensitively. Since PQQ-GDH can react with various electron acceptors at a relatively high rate constant,¹⁶ it is expected that the signal amplification of both DHN and Cat can be achieved in a similar manner. In this study, we prepared an amperometric biosensor by modifying a carbon electrode with PQQ-GDH. The simultaneous determinations of DHN and Cat were performed with the biosensor.

Experimental

Reagents and chemicals

PQQ-GDH (GLD-321) was purchased from Toyobo (Osaka, Japan) and used as received. 1,2-Dihydroxynaphthalene was purchased from Tokyo Chemical Industry (Tokyo, Japan). Catechol and other reagents were from Wako (Osaka, Japan). All chemicals were of reagent grade and used without further purification.

Preparation of biosensor

A plastic formed carbon electrode (PFCE, BAS Inc., 3-mm diameter disk) was polished with a slurry of 0.05- μm alumina. A 5- μL aliquot of 2 U μL^{-1} PQQ-GDH solution was applied on the polished surface of PFCE. The solvent was evaporated, and then the electrode surface was covered with a dialysis membrane (Wako, cutoff molecular weight of 14000, 20- μm thick in the dry state). The membrane was overlaid with a nylon net to give it physical strength. When not in use, it was stored in a refrigerator at 4°C with the surface of the electrode soaked in a pH 7.0 phosphate buffer.

Electrochemical measurements

Cyclic or linear sweep voltammetry and constant-potential amperometry were carried out in buffer solutions with various pH values, ionic strengths, and D-glucose concentrations. The test solution was stirred by a magnetic stirrer at 100 rpm. A platinum coil and an Ag/AgCl (0.1 M KCl) electrode were used as the counter and reference electrodes, respectively. Voltammograms were recorded by a potentiostat HA-1010mM1A (Hokuto Denko, Tokyo, Japan) with a laboratory-made program. All of the experiments were performed at 25.0 $\pm$ 0.5°C.

Results and Discussion

Enzyme-catalyzed signal amplification

Figure 1 shows cyclic voltammograms of 1.0 μM DHN (A) and Cat (B) at a PQQ-GDH-modified PFCE or unmodified PFCE in a pH 7.0 phosphate buffer with 0.1 M D-glucose. In the absence of PQQ-GDH (broken curves), almost no anodic current signals of 1.0 μM DHN and Cat were seen. When the electrode was modified with PQQ-GDH, a sigmoidal-shaped voltammogram of DHN was observed with a much larger anodic limiting current (red curve). Although the limiting current of

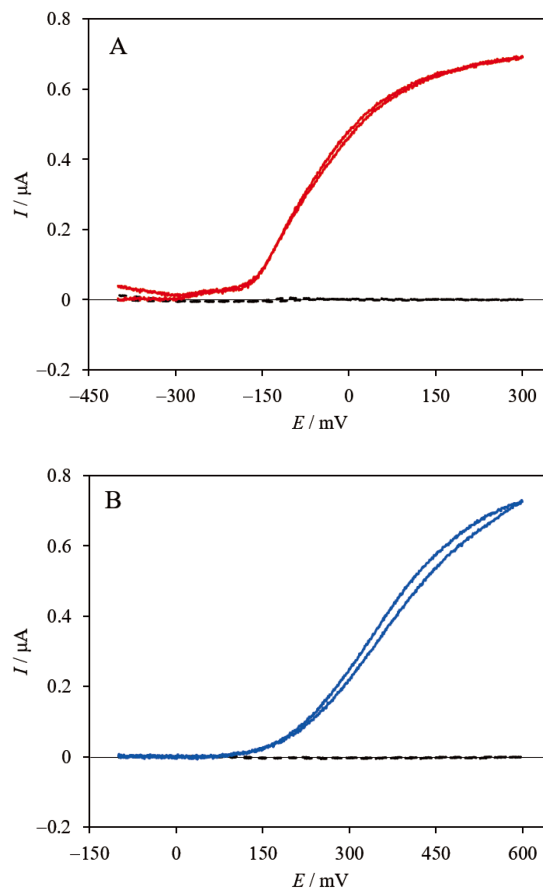


Fig. 1 Cyclic voltammograms of (A) 1.0 μM DHN and (B) 1.0 μM Cat in 0.1 M phosphate buffer (pH 7.0) containing 0.1 M D-glucose at the PQQ-GDH-modified PFCE (solid curves) or unmodified PFCE (broken curves). Scan rate, 5 mV s^{-1} .

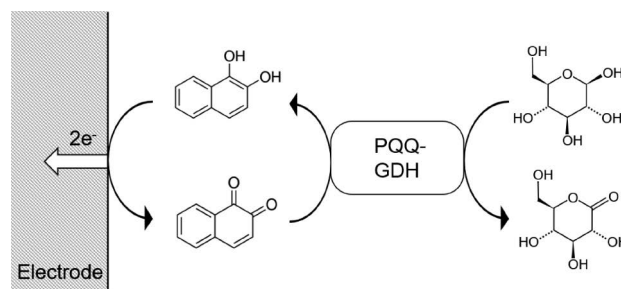


Fig. 2 Signal amplification by PQQ-GDH-catalyzed redox cycling in the presence of DHN and D-glucose.

Cat was unclear, the current signal was amplified significantly (blue curve). These results can be attributed to enzyme-catalyzed signal amplification, in which the electrochemical oxidation of DHN or Cat was coupled with the enzymatic reduction of 1,2-naphthoquinone or *o*-quinone to regenerate DHN or Cat, respectively, in the presence of D-glucose (Fig. 2). In our previous study,¹⁴ the limiting current of Cat was more clearly observed when PQQ-GDH was dissolved in the test solution. The broader wave of Cat in the present study may be due to electrode fouling by the high concentration of PQQ-GDH immobilized on the surface of the electrode.

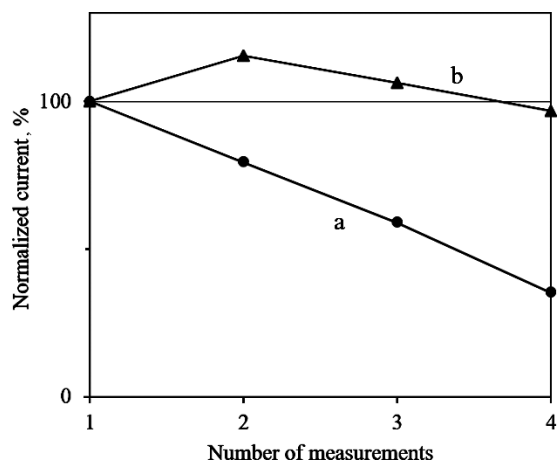


Fig. 3 Limiting currents of DHN in repeated measurements at (a) 5.0 μM and (b) 0.20 μM . The current values are normalized to the first measurement.

Figure 3 shows the limiting currents of DHN in repeated measurements. The current values are normalized to the first measurement. Curves a and b represent the current signals at DHN concentrations of 5.0 and 0.20 μM , respectively. There was a marked tendency for the current signal to decrease at relatively high concentrations of DHN ($>1 \mu\text{M}$), whereas it was not obvious at lower concentrations. This tendency was more significant as the applied amount of PQQ-GDH or the concentration of D-glucose was increased. After repeated measurements, a brownish deposit was found on the surface of the electrode, probably due to the formation of an insoluble dimer or polymer during the electrochemical oxidation of DHN.¹⁷ This product may cause electrode fouling, and thus reduce the current. It is known that Cat also undergoes dimerization or polymerization,¹⁸ but the influence on the limiting current was negligible in our measurements.

Calibration curves

Figure 4A shows the current (I)-time (t) curve at a constant potential of 100 mV upon the successive addition of DHN. The anodic current increased after each addition, and then an almost constant current was obtained, which can be regarded as steady state, although there seemed to be a slight drift upward. Figure 4B is a calibration curve of the steady-state currents (I_{ss}) of DHN plotted against the molar concentration of DHN ([DHN]). I_{ss} was proportional to [DHN] up to 1.0 μM , suggesting that these concentrations were sufficiently lower than the Michaelis constant of PQQ-GDH for DHN. The equation of the regression line in Fig. 4B was

$$I_{ss}/\text{nA} = (0.52 \pm 0.01) \times [\text{DHN}]/\text{nM} - (8 \pm 2). \quad (1)$$

Here, and in the following, the values with “ $\pm$ ” indicate the standard deviations obtained by regression analysis.

In a similar manner, the I - t curve was taken at the constant potential of 600 mV upon the successive addition of Cat. I_{ss} was proportional to the molar concentration of Cat ([Cat]) up to 1.0 μM . The equation of the regression line was

$$I_{ss}/\text{nA} = (0.59 \pm 0.03) \times [\text{Cat}]/\text{nM} - (4 \pm 2). \quad (2)$$

Cat gave a slightly larger sensitivity than DHN. From the slopes and the standard deviations of the regression, the limits of

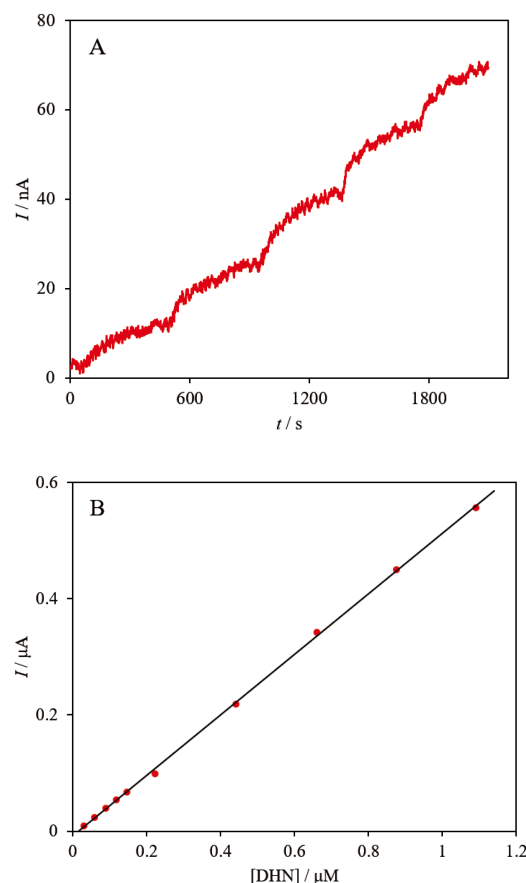


Fig. 4 (A) I - t curve at $E = 100 \text{ mV}$ upon the successive addition of 30 nM DHN. (B) Plot of I_{ss} against [DHN]. The solid line is the regression line.

detection (LOD) were estimated to be 3 nM for both DHN and Cat. These LOD values are comparable to the results of our previous reports for Cat (8 nM)¹⁴ and 2,6-dimethylhydroquinone (1 nM).¹⁵

The present biosensor was stable at between pH 6 and 8. Within this pH range, the sensitivity increased as the test solution became more alkaline, according to the pH dependence of the activity of PQQ-GDH.¹⁹ The stability was found upon the addition of NaCl up to 10%, whereas the sensitivity was practically independent of the NaCl concentration up to 5%, and significantly reduced at 10%, probably due to the inactivation of PQQ-GDH by salt. From these results, it can be said that the present biosensor is directly applicable to a microbial cell suspension, when the pH value is around neutrality and the salt concentration is at the level of brackish water. It is difficult to conduct a direct measurement at seawater salinity.

Simultaneous determinations of DHN and Cat

The anodic waves of DHN and Cat were far apart in the applied potential, as shown in Fig. 1. We examined the voltammetric behavior of the biosensor in mixed solutions of DHN and Cat, expecting simultaneous determinations. Figure 5A shows linear sweep voltammograms for different concentrations of DHN at a fixed concentration of Cat (0.20 μM); Fig. 5B shows the concentration dependence of the currents at the applied potential of 100 mV ($I_{100\text{mV}}$) and the difference between those of 600 and 100 mV ($I_{600\text{mV}} - I_{100\text{mV}}$),

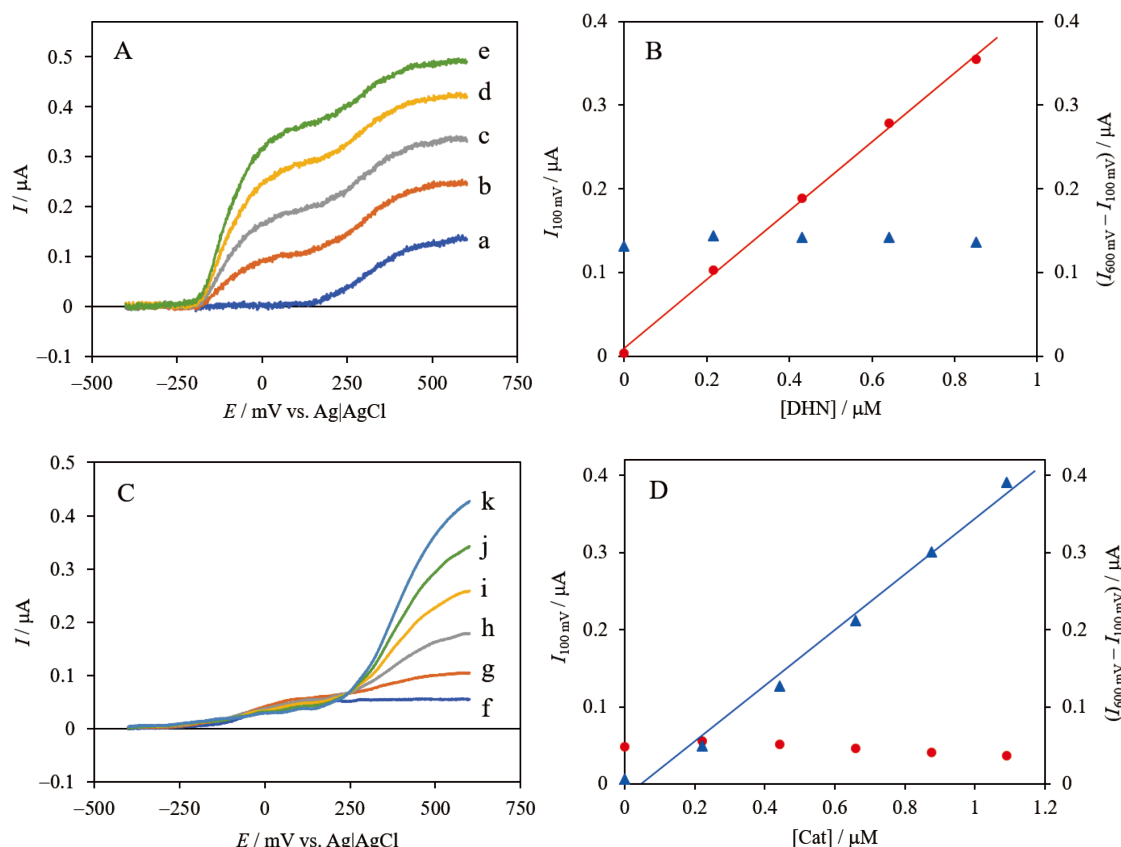


Fig. 5 (A) Linear sweep voltammograms for different concentrations of DHN at a fixed concentration of Cat (0.20 μM). [DHN] = (a) 0, (b) 0.21, (c) 0.43, (d) 0.64, and (e) 0.85 μM. (B) Dependence of $I_{100\text{mV}}$ (red circles, left ordinate) and $I_{600\text{mV}} - I_{100\text{mV}}$ (blue triangles, right ordinate) on [DHN]. (C) Linear sweep voltammograms for different concentrations of Cat at a fixed concentration of DHN (0.20 μM). [DHN] = (f) 0, (g) 0.22, (h) 0.44, (i) 0.66, (j) 0.88, and (k) 1.09 μM. (D) The dependence of $I_{100\text{mV}}$ (red circles, left ordinate) and $I_{600\text{mV}} - I_{100\text{mV}}$ (blue triangles, right ordinate) on [Cat]. Scan rate; 5 mV s⁻¹.

which correspond to the oxidation of DHN and Cat, respectively. The $I_{100\text{mV}}$ values (red circles) showed good proportionality to the DHN concentration in the range from 0.2 to 1 μM, whereas the $I_{600\text{mV}} - I_{100\text{mV}}$ values (blue triangles) were almost constant, regardless of the concentration of DHN. On the contrary, Fig. 5C shows linear sweep voltammograms for different concentrations of Cat at a fixed concentration of DHN (0.20 μM); Fig. 5D shows the concentration dependence of $I_{100\text{mV}}$ and $I_{600\text{mV}} - I_{100\text{mV}}$. The $I_{600\text{mV}} - I_{100\text{mV}}$ values (blue triangles) showed good proportionality to the Cat concentration in the range from 0.2 to 1 μM, whereas the $I_{100\text{mV}}$ values (red circles) were almost constant, regardless of the concentration of Cat. Thus, a simple way for simultaneous voltammetric determinations of DHN and Cat has been established. The slopes of the calibration curves in Figs. 5B and 5D were somewhat smaller than those of Eqs. (1) and (2). There may be an influence of the competitive inhibition between DHN and Cat.

Response to other metabolites of PAHs

It is known that gentisic acid (2,5-dihydroxybenzoic acid) is produced by the further degradation of naphthalene.¹² This compound also has the quinone structure and can be readily oxidized. When 1.0 μM gentisic acid was measured by the biosensor in the presence of glucose, a very small anodic current signal, which was comparable to the unamplified response of DHN or Cat in Fig. 1, was observed, showing that the enzyme-

catalyzed signal amplification did not work well, probably because the electron transfer from PQQ-GDH to the quinone form of gentisic acid was slow. Accordingly, it was difficult to detect gentisic acid at low concentrations by the present method.

2,3-Dihydroxynaphthalene is known to be a primary metabolite in anthracene degradation.¹³ This has the catechol structure and can also give an electrochemical signal. However, the anodic current signal obtained by the biosensor was very small, showing that it was not amplified well, as in the case of gentisic acid. 2,3-Dihydroxynaphthalene is an isomer of (1,2-)DHN, and the only difference in structure is the position of hydroxy groups. It is interesting that PQQ-GDH is selective for 1,2-substituted naphthalene.

Conclusions

A biosensor used to monitor the PAH degradation has been constructed. We are planning to apply this to a liquid culture medium or a cell-free extract of PAH-degrading bacteria. When naphthalene or phenanthrene is added, it is expected that DHN is detected in the early stage of the degradation, and subsequently Cat is observed. Simultaneous measurements by the biosensor will give reaction curves for both metabolites, which will characterize the degradation activity of the bacteria.

Of course, many other metabolites can also be produced from

PAHs. Although the measurements of gentisic acid and 2,3-dihydroxynaphthalene were unsuccessful by the present method, it is still possible to develop another sensitive electrochemical method for them. We are now seeking other redox enzymes which can amplify the current signal of those metabolites.

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