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

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Abstract

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 the 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 determination of these compounds was 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 μM).
Introduction

Mangrove forests have unique ecosystem with rich biodiversity in intertidal zone. The ecosystem is formed in flat coastal area, which is 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 one of the most serious pollutants, because of their persistent nature. One possible approach to remove these pollutants is bioremediation, in which PAHs are degraded by microorganisms. Many reports have appeared on the PAH-degrading bacteria isolated from mangrove sediments.

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, gas chromatography, gas chromatography – mass spectrometry, 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 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 the 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 does not give any signal in usual electrochemical measurements. Reshetilov et al. 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 dioxygenase enzyme.\textsuperscript{12,13} By further degradation, catechol (Cat) is also formed.\textsuperscript{12} It is possible to develop a new method of evaluation of PAH-degrading ability, based on the detection of these electroactive metabolites.

One feature required for the analysis of this purpose is the 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 hydroxyl radical.\textsuperscript{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,\textsuperscript{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 determination of DHN and Cat was 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 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 14,000, 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, ionic strength, 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 the experiments were performed at 25.0 ± 0.5 °C.
Results and Discussion

Enzyme-catalyzed signal amplification

Figure 1 shows the cyclic voltammograms of 1.0 μM DHN (A) and Cat (B) at the 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 much larger anodic limiting current (red curve). Although the limiting current of Cat was unclear, the current signal was amplified significantly (blue curve). These can be attributed to the 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,\textsuperscript{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 the electrode fouling by the high concentration of PQQ-GDH immobilized on the surface of the electrode.

Figure 3 shows the limiting currents of DHN in repeated measurements. The current values are normalized to the first measurement. The curves a and b represent the current signals at the 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 μM), whereas it was not obvious at lower concentrations. The tendency was more significant as the applied amount of PQQ-GDH or the concentration of D-glucose was increased. After the repeated measurements, brownish deposit was found on the
surface of the electrode, probably due to the formation of insoluble dimer or polymer during the electrochemical oxidation of DHN.\textsuperscript{17} This product may cause the electrode fouling and thus reduce the current. It is known that Cat also undergoes the dimerization or polymerization,\textsuperscript{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 the constant potential of 100 mV upon the successive addition of DHN. The anodic current increased after each addition, and then almost constant current was obtained which can be regarded as steady state, although there seemed to be a slight drift upward. Figure 4B is the calibration curve of the steady-state currents ($I_{ss}$) of DHN plotted against the molar concentration of DHN ([DHN]). The $I_{ss}$ 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:

\[
\frac{I_{ss}}{nA} = (0.52 \pm 0.01) \times \left(\frac{[\text{DHN}]}{nM}\right) - (8 \pm 2). \tag{1}
\]

Here and in the following, the values with “±” indicate the standard deviations obtained by the 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. The $I_{ss}$ was proportional to the molar concentration of Cat ([Cat]) up to 1.0 μM. The equation of the regression line was:

\[
\frac{I_{ss}}{nA} = (0.59 \pm 0.03) \times \left(\frac{[\text{Cat}]}{nM}\right) - (4 \pm 2). \tag{2}
\]
Cat gave slightly larger sensitivity than DHN. From the slopes and the standard deviations of the regression, the limits of 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 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 at the addition of NaCl up to 10 \(\%\), whereas the sensitivity was practically independent of 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 applicable directly to a microbial cell suspension, when the pH 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 determination 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 the simultaneous determination. Figure 5A shows the linear sweep voltammograms for different concentrations of DHN at a fixed concentration of Cat (0.20 \(\mu\)M), and Fig. 5B shows the concentration dependence of the currents at the applied potential of 100 mV \((I_{100mV})\) and the difference between those of 600 mV and 100 mV \((I_{600mV} − I_{100mV})\), which correspond to the oxidation of DHN and Cat, respectively. The \(I_{100mV}\) (red circles) showed a good proportionality to the DHN concentration in the range from 0.2 \(\mu\)M to 1 \(\mu\)M, whereas the \(I_{600mV} − I_{100mV}\) (blue
triangles) were almost constant, regardless of the concentration of DHN. On the contrary, Fig. 5C shows the linear sweep voltammograms for different concentrations of Cat at a fixed concentration of DHN (0.20 μM), and Fig. 5D shows the concentration dependence of $I_{100\text{mV}}$ and $I_{600\text{mV}} - I_{100\text{mV}}$. The $I_{600\text{mV}} - I_{100\text{mV}}$ (blue triangles) showed a good proportionality to the Cat concentration in the range from 0.2 μM to 1 μM, whereas the $I_{100\text{mV}}$ (red circles) were almost constant, regardless of the concentration of Cat. Thus, a simple way of simultaneous voltammetric determination of DHN and Cat has been established. The slopes of the calibration curves in Fig. 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. 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-Dihydroxynaphtalene is known as a primary metabolite in anthracene degradation. This has the catechol structure and can also give 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-Dihydroxynaphtalene 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 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. The simultaneous measurement by the biosensor will give the 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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Figure Captions

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.

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.

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

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) The dependence of $I_{100mV}$ (red circles, left ordinate) and $I_{600mV} - I_{100mV}$ (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 $I_{100mV}$ (red circles, left ordinate) and $I_{600mV} - I_{100mV}$ (blue triangles, right ordinate) on [Cat]. Scan rate; 5 mV s⁻¹.
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