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Highly sensitive flow-biosensor for toxic phenolic compounds using tyrosinase and acridine orange-adsorbed carbon felt

WANG Yue[1,2], HASEBE Yasushi[1,3,*]

1. Department of Material Science and Engineering, Saitama Institute of Technology, Fukaya 369-0293, Japan. E-mail: q8003ctz@sit.jp
2. School of Chemical Engineering, University of Science and Technology Liaoning, Anshan 114501, China
3. Department of Life Science and Green Chemistry, Saitama Institute of Technology, Fukaya 369-0293, Japan

Abstract

Tyrosinase (TYR, EC 1.14.18.1) was physically adsorbed onto a carbon felt (CF) together with acridine orange (AO). Coadsorption of AO was essential to prevent the denaturation of the TYR at the CF surface. The resulting TYR and AO-coadsorbed CF (TYR/AO-CF) was successfully utilized as a detection unit of novel and highly sensitive amperometric flow-biosensor for toxic chlorophenol compounds. Standard solutions of phenolic compounds (200 μL) were injected, and the cathodic peak currents due to the reduction current of *o*-quinones produced by the TYR-catalyzed oxidation (phenolase activity) were detected at the applied potential of –50 mV vs. Ag/AgCl. In this reaction, the electrochemically generated catechol compounds from *o*-quinones are re-oxidized repeatedly by catecholase activity of the TYR, leading to a sufficient amplified signal. The TYR/AO-CF exhibited much higher selectivity toward *p*-chlorophenol as compared with other chlorophenol compounds. When 0.1 mol/L phosphate buffer (pH 7.0) was used as a carrier at flow rate of 3.0 mL/min, cathodic peaks for *p*-chlorophenol was linear in the concentration range between 0.1 and 10 μmol/L (sensitivity: 1.41(mA·L)/mmol) with sampling rate (30 samples/h), and the detection limit of *p*-chlorophenol was found to be 2.13×10^{-8} mol/L (S/N = 3. The ratio of signal and noise is 3). The TYR/AO-CF kept more than 80% of original activity after the storage in 0.1 mol/L phosphate buffer (pH 7.0) containing 0.2 mmol/L AO at 4°C.

Key words: flow-biosensor; carbon felt; tyrosinase; acridine orange; phenol compounds

Introduction

Phenolic compounds are important contaminations in ground water and surface water (Vincent *et al*., 1991), causing problems for our living environment and showing adverse effects on animals and plants. The determination of phenol compounds is of great importance due to their toxic and persistency in the environment (Heath, 1987; Hennion *et al*., 1994; Svitel and Miertus, 1998). Many analytical techniques have been used for monitoring phenols, such as colorimetry (Castillo *et al*., 1997), gas chromatography (Crespín *et al*., 2002), liquid chromatography (Muna *et al*., 2004), and capillary electrophoresis (O'Neill *et al*., 2001). However, these methods usually suffer from complicated samples pre-treatment and lack of sensitivity. Therefore, the sensitive, rapid, and precise determination of phenols and its derivatives are of growing interest in environmental control and protection.

A carbon felt (CF) is a microelectrode ensemble of micro carbon fiber (ca. 7 μm diameter), and possesses a random three-dimensional structure (Fig. 1). The CF has high surface area (estimated to be 0.3–30 m^2/g) and shows a high conductivity and electrolytic efficiency. In addition, low diffusion barrier of porous CF facilitates mass transport. Thus, the CF is useful as a working electrode unit of electrochemical flow detector (Hasebe *et al*., 2006).

In general, highly specific catalytic activity of enzymes allows convenient and simple analysis of various species by combining with electroactive-materials. Thus, enzyme-immobilized CF would allow highly selective flow-detection of enzyme-substrates (Hasebe *et al*., 2007). However, simple and convenient immobilization strategy of biomolecules onto the CF surface has not completely been established so far in our best knowledge. Therefore, the development of immobilization method of enzymes onto the CF surface is attractive research topic.

Tyrosinase (TYR: EC 1.14.18.1), also known as polyphenol oxidase, is now a well-characterized enzyme. Tyrosinase contains two binuclear copper-containing active sites (Yong *et al*., 1990). TYR catalyzes two distinct oxidation reactions as shown in Scheme 1. In cycle I, TYR accomplishes the oxidation of monophenols by oxygen as it passes through four enzyme states (E_{deoxy}, E_{oxy}, $E_{oxy\text{-}M}$, and $E_{met\text{-}D}$); in cycle II, *o*-diphenols are oxidized as the enzyme passes through five enzyme states (E_{deoxy}, E_{oxy}, $E_{oxy\text{-}D}$, E_{met}, and $E_{met\text{-}D}$). The two cycles lead to the formation of *o*-quinones, which spontaneously react with each other to form oligomers (Wang *et al*., 2002; Sánchez-Ferrer *et al*., 1995; Fenoll *et al*., 2001; Seo *et al*., 2003). Therefore, TYR is useful as recognition element for both

* Corresponding author. E-mail: hasebe@sit.ac.jp

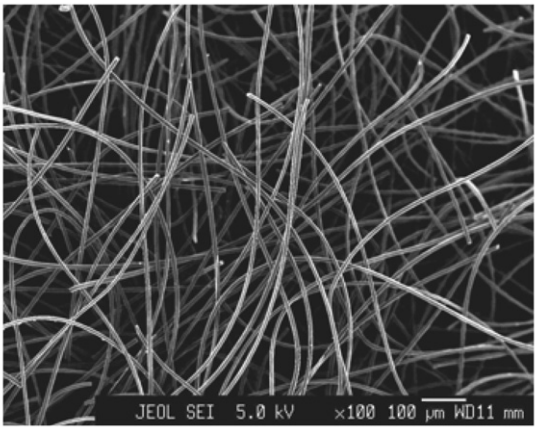


Fig. 1 SEM image of the CF.

phenolic compounds and catechol compounds.

In this study, very simple and novel enzyme immobilization strategy onto the CF surface was proposed. The TYR and acridine orange (AO) were simply co-adsorbed onto the CF surface from their mixed aqueous solution, and the resulting TYR and AO-coadsorbed CF (TYR/AO-CF) was successfully utilized as a novel bioelectrocatalytic sensing unit of flow-biosensor system for the determination of toxic phenolic compounds. Coadsorption of AO together with TYR is essential to prevent the denaturation of the TYR at the CF surface, and the catalytic activity of adsorbed TYR is significantly dependent on the AO concentration of the TYR/AO adsorption solution. The concentration of AO was optimized, and the analytical performance characteristics of the biosensor with respect to sensitivity, selectivity and stability were evaluated.

1 Experimental

1.1 Reagents

Tyrosinase (TYR, polyphenol oxidase, EC 1.14.18.1, 5370 unit/mg from mushroom) was purchased from Sigma Co. (USA) and used as received. Acridine orange, phenol, catechol, 2,4-, 2,6-, and 3,4-dichlorophenols were obtained from Wako Pure Chemicals (Japan). 2,3-Dichlorophenol was obtained from Aldrich Co. (USA), *p*-, *o*-, and *m*-chlorophenols and *p*-cresol were from Tokyo Kasei Kogyo (Japan). All other chemicals were analytical grade, and used without further purifications. Standard solutions of phenolic compounds were prepared by dissolving each reagent in water.

1.2 Preparation of enzyme-immobilized CF

CF was cut into 10 mm × 3 mm × 3 mm in size, and washed with distilled water by ultra-sonication (As one Ltd., Japan) for 10 min, and was dried in vacuum (As one Ltd., Japan). Then the CF was immersed into AO and TYR-mixed aqueous solution for 15 min. The prepared TYR/AO-CF was used as working electrode unit of the electrochemical flow-detector.

1.3 Electrochemical measurements

Electrochemical measurements were performed with the electrochemical analyzer (CHI 6122A, CH Instruments, Inc., USA) in connection with a computer. Cyclic voltammetry (CV) and electrochemical impedance spectrometry (EIS) in batch mode were carried out at room temperature with a conventional three-electrode system equipped with a platinum wire counter electrode (diameter 1 mm), an Ag/AgCl (3 mol/L NaCl) reference electrode, and the CF working electrode (with platinum lead wire, diameter 0.5 mm) in deoxygenized 10 mL of phosphate buffer (0.1 mol/L, pH 7.0).

Flow-injection determination was carrier out using FIA system consisted with double plunger pump (DMX-2000T, SNK, Co., Japan) with six-way injection valve and the TYR/AO-CF-based electrochemical flow-detector. Air-saturated 0.1 mol/L phosphate buffer (pH 7.0) was used as a carrier at 3.0 mL/min, and the standard solutions of phenol derivatives (200 μL) were injected. The cathodic peak current due to the reduction of *o*-quinones produced by the TYR-catalyzed oxidase reaction was detected at applied potential of –50 mV vs. Ag/AgCl.

2 Results and discussion

2.1 Effect of AO on the catalytic activity of adsorbed TYR

In general, adsorbed enzymes are denatured at highly hydrophobic surfaces due to the deformation of the active conformation of the enzyme. The CF surface has much hydrophobic region. Therefore, the enzyme-adsorbed CF usually cannot be used for biotechnology applications, because adsorbed enzyme at the CF surface tends to lose their catalytic activities. In this study, we found that coadsorption of AO and TYR from AO-TYR mixed aqueous solution is much effective to prevent the denaturation of the TYR at the CF surface.

Figure 2 shows the effect of AO concentration in adsorption solution upon the cathodic peak current for 30 μmol/L *p*-chlorophenol. When only TYR was adsorbed onto CF in

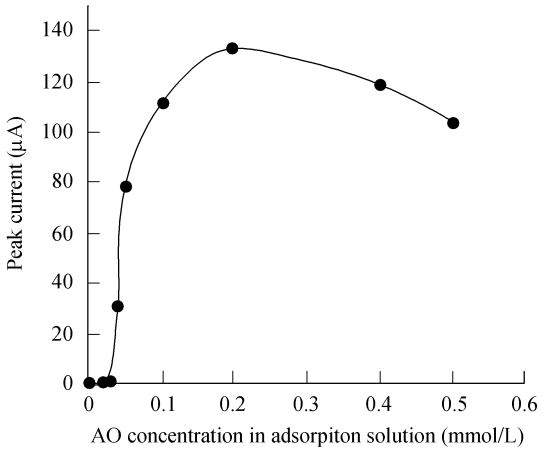
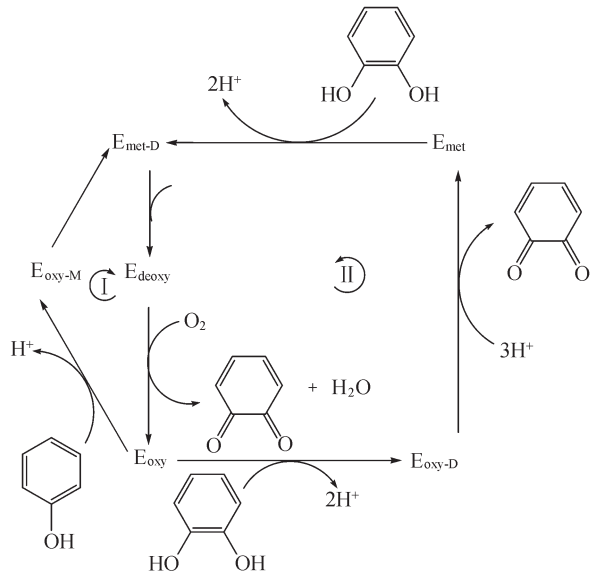


Fig. 2 Effect of AO concentration in adsorption solution on the peak current response to *p*-chlorophenol (30 μmol/L).

the absence of AO, no apparent current was observed. In contrast, the current response significantly increased with increasing in AO concentration in the adsorption solution ranging from 0.03 to 0.1 mmol/L, and the maximum response was obtained at AO concentration of 0.2 mmol/L. This result clearly indicates that the coadsorption of AO together with the TYR is essential for the generation of catalytic activity of the adsorbed TYR.



Scheme 1 Catalytic cycles for the (I) hydroxylation of monophenols (M) and (II) dehydrogenation of *o*-diphenols (D) to *o*-quinones by tyrosinase (TYR) (Wang *et al*., 2002)

To elucidate the surface characteristics of the TYR-adsorbed CF, we measured CV and EIS using 1 mmol/L $[Fe(CN)_6]^{4-/3-}$ as electrochemical probe. Figure 3 shows the CV (A) and EIS (B) of the TYR-CF and TYR/AO-CF. The surface resistance of the TYR-CF was much higher than that of the TYR/AO-CF, indicating that the TYR is strongly adsorbed onto the CF. Therefore, it is clear that most of the adsorbed TYR at the CF surface lost its catalytic activity. This result is well consistent with the previous report in which the protein is strongly adsorbed onto the carbon nanotube and structure of the protein is considerably changed due to the strong interaction between the protein and the carbon nanotube surface (Karajanagi *et al*., 2004). In contrast, the TYR adsorbed together with AO retained its catalytic activity even at the CF surface. Some special interaction between AO and TYR may contribute to increase of a resistance of the TYR against the conformational change of the TYR at the CF surface. However, more systematic studies are required to elucidate this assumption.

2.2 Flow-biosensor characteristics

As mentioned above (Introduction section: paragraph 4), the TYR has two catalytic activities (i.e., phenolase activity and catecholase activity). In fact, the TYR/AO-CF exhibited higher response to catechol (sensitivity: 5.40 (mA·L)/mmol) than *p*-chlorophenol (sensitivity: 1.41 (mA·L)/mmol). However, the analysis of catechol compounds is beyond the purpose and the scope of the present work, thereby, we focused on the highly toxic chlorophenol compounds. In general, the TYR shows a broad specificity toward various mono- and di-phenolic compounds. Thus we checked the selectivity toward various mono- and di-chlorophenol compounds. The results are summarized in Table 1. The TYR/AO-CF-based flow biosensor exhibited the highest selectivity toward *p*-chlorophenol as compared with *m*- and *o*-chlorophenols and various dichlorophenols. This selectivity is probably originating from the substrate specificity of the TYR.

Table 1 Substrate selectivity of the sensor for toxic chlorophenol derivatives (phenol conc. 30 μmol/L)

Phenol	Peak current (μA)	Relative response (%)
p-CP	133.2	100
p-Cresol	61.9	47
m-CP	1.7	1.3
o-CP	0.3	0.23
2,3-DCP	0.4	0.28
3,4-DCP	0.3	0.22
2,6-DCP	0.2	0.17
2,4-DCP	0.1	0.09

CP: chlorophenol; DCP: dichlorophenol.

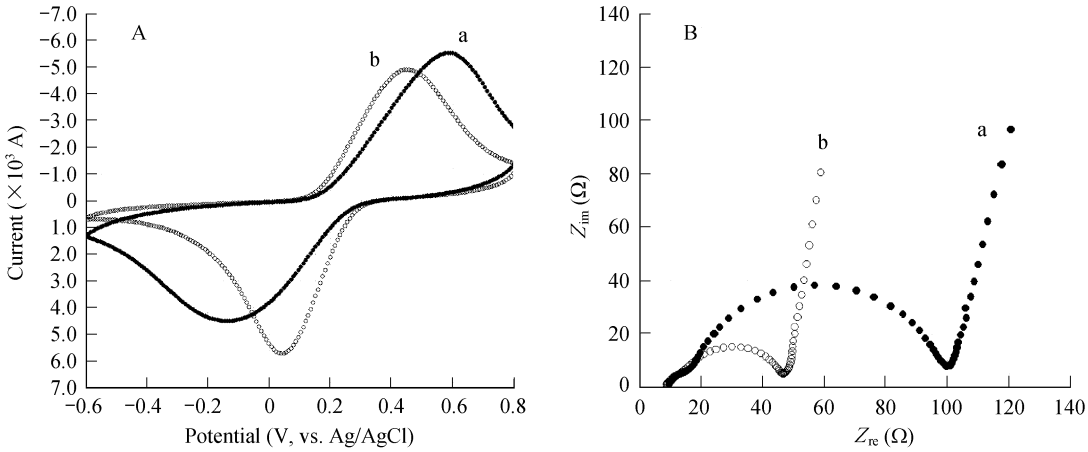


Fig. 3 CV (A) and EIS (B) of the TYR-CF (a) and TYR/AO-CF (b). Deoxygenated 0.1 mol/L phosphate buffer containing 1 mmol/L $K_4Fe(CN)_6$ and $K_3Fe(CN)_6$ was used as an electrolyte. Z_{re}: real impedance; Z_{im}: imaginary impedance.

Figure 4 shows the typical flow-injection peaks toward various concentrations of *p*-chlorophenol. Peak current responses were highly reproducible. At carrier flow rate of 3.0 mL/min, sampling rate was ca. 30 samples/h. Figure 5 shows a calibration curve of *p*-chlorophenol. The cathodic peak currents linearly increased in the concentration range from 0.1 to 10 μmol/L (sensitivity: 1.41 (mA·L)/mmol), and the detection limit was found to be 2.13×10^{-8} mol/L (S/N = 3, the ratio of signal and noise is 3).

2.3 Sensor stability and storage condition

The CF was also studied for the enzyme stability stored both in phosphate buffer (PB) solution as well as AO-containing PB solution at 4°C in refrigerator. The stability of the enzyme was monitored after an interval of 10 days to a 30 μmol/L *p*-chlorophenol sample for 1 month (Fig. 6). It was observed that the TYR/AO-CF retained almost 80% of its initial activity when stored in AO-containing buffer solution for 1 month. However in PB solution, the TYR/AO-CF lost 99% of its initial enzyme activity after 20 days' storage. This result also indicates that AO plays an interesting role as a stabilizer of the adsorbed TYR.

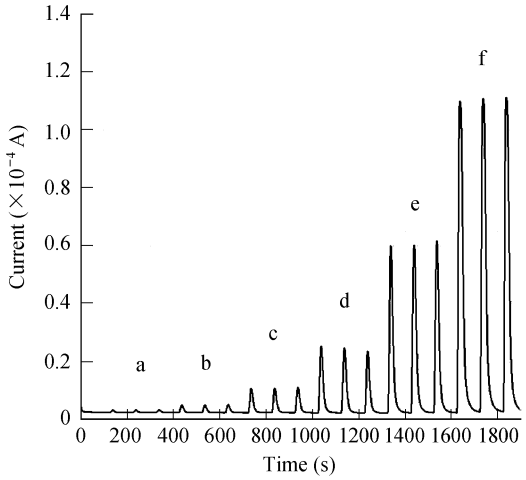


Fig. 4 Typical flow-injection peaks to different *p*-chlorophenol concentrations. (a) 0.1 mol/L; (b) 0.3 mol/L; (c) 1 mol/L; (d) 3 mol/L; (e) 10 mol/L; (f) 30 mol/L.

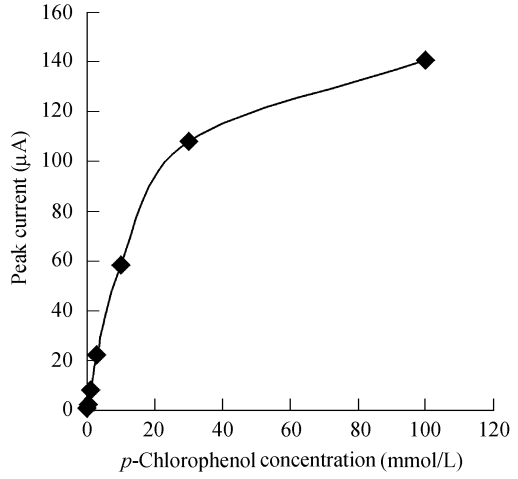


Fig. 5 Calibration curve of *p*-chlorophenol.

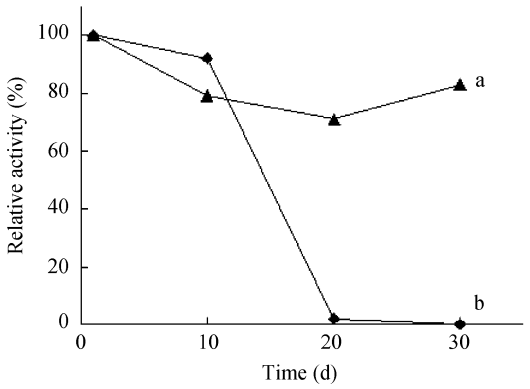


Fig. 6 Storage stabilities of the TYR/AO-CF. The TYR/AO/CF was kept in phosphate buffer (pH 7.0) with (a) and without (b) 0.2 mmol/L AO. The CF was kept for 1 month and was tested every 10 d using 30 mol/L *p*-chlorophenol as a substrate.

3 Conclusions

We developed novel and highly sensitive TYR/AO-CF-based flow-biosensor system for the determination of highly toxic chlorophenol compounds. Coadsorption of AO was essential not only to prevent the denaturation of the TYR at the CF surface but also to stabilize the adsorbed TYR. This enzyme immobilization method is very simple, and has potential applicability to not only biosensors but also bioreactors and bio-fuel cells.

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