Highly Sensitive Detection of 4-Chlorophenol by a Flow Injection Biosensor System Based on Substrate Recycling

Toshio Yao * and Kazuya Kotegawa

Department of Applied Chemistry, Graduate School of Engineering, Osaka Prefecture University, 1-1 Gakuencho, Sakai, Osaka 599-8531, Japan

Abstract

A flow injection biosensor system was proposed for the highly sensitive detection of 4-chlorophenol (4-CP). The tyrosinase-based biosensor used in this analytical system responded sensitively to 4-CP, based on the bioelectrocatalytic recycling occurring effectively at the tyrosinase membrane / glassy carbon interface of the biosensor, because 4-chlorobenzoquinone produced from 4-CP by the tyrosinase catalyzed reaction was an electrochemically reversible redox species. The signal current was linearly related to the 4-CP concentrations in a dynamic range of 5×10^{-9}- 2×10^{-6} M for a 20 μl injection; the slope and the y-intercept of the straight line were 279 nA μM^{-1} and 3.2 nA, respectively. The detection limit was 3 nM (S/N=3). Among many chlorophenols, 3-chlorophenol and 2,3-, 2,4-, and 3,4-dichlorophenols interfered in some degree (< 5%) with the measurement of 4-CP.

Keywords Flow injection, biosensor, tyrosinase, 4-chlorophenol, amplification, substrate recycling, amperometric detection

1. Introduction

Many chlorophenols have been widely used as pesticides, herbicides, and wood preservatives. Among these chlorophenols, 4-chlorophenol (4-CP) is also by-product of many industrial preparations, contaminates the ecosystem, and accumulates in the food chain of organisms. Therefore, there is a great interest in developing simple, sensitive, and accurate analytical method for measuring 4-CP. A variety of methods for determining chlorophenols have been available, by gas chlomatography [1, 2], liquid chromatography [3-6], GC-MS [7, 8], LC-MS [9], capillary electrophoresis [10], and enzyme immunoassay [6, 11]. These methods, however, involve some complicated and time consuming procedures and require expensive equipments. They are also not suitable for simple and rapid monitoring.

Therefore, it is of a great importance to develop the sensors which respond selectively to such chlorophenols, in the field of environmental and food analysis. A few research groups have developed microbial sensors based on *Escherichia coil* or *Pseudomonas fluorescens* [12] and recombinant bioluminescent bacteria [13]. However, the drawbacks of such sensors were lack of selectivity and sensitivity for the detection of 4-CP at the low concentration levels. In contrast, preparations of electrode-based biosensors are typically simple, low in cost, and fairly high in sensitivity. The use of phenol-oxidizing enzymes in biosensor construction was one interesting approach to achieve selective and sensitive measurement for chlorophenols. Most of enzyme-based biosensor for phenolic compounds is based on the use of tyrosinase [14, 15], laccase [16], or horseradish peroxidase [17, 18]. In this work, these enzymes have been attempted for the biocatalytic oxidation of 4-CP and we found out that tyrosinase can oxidize effectively 4-CP to the corresponding quinone.

In this paper, we describe a flow injection biosensor system for the highly sensitive and selective detection of 4-CP. Sensor response involves amplification based on the substrate recycling occurring at the interface of tyrosinase membrane and electrode of the biosensor. As a result, tyrosinase-based biosensor responded sensitively to 4-CP at a nM concentration level in a flow injection system.

*Corresponding author.
E-mail: yao@chem.osakafu-u.ac.jp

2. Experimental

2.1. Reagents

The enzymes used, tyrosinase (EC 1.14.18.1; 4800 U mg^{-1} of solid from mashroom) and peroxidase (EC 1.11.1.7; 66 U mg^{-1} of solid from horseradish) were obtained from Sigma and laccase (EC 1.10.3.2; 540 U ml^{-1} of solution from *Pycnoporus coccineus*) was from Funakoshi. 2-, 3-, and 4-Chlorophenols, 2,3-, 2,4-, 2,5-, 2,6-, 3,4-, and 3,5-dichlorophenols, 2,3,4-, 2,3,5-, 2,3,6-, 2,4,5-, and 2,4,6-trichlorophenols, and pentachlorophenol were obtained from Aldrich. All other reagent grade chemicals were obtained from Wako. They were used as received. Phosphate buffer used as the carrier solution was prepared from sodium dihydrogenphosphate. Distilled water purified using a Millipore Milli Q system (Nippon Millipore) was used throughout.

2.2. Construction of enzyme-based biosensors

A glassy carbon (GC) disk electrode (5 mm in diameter) and an Eicom thin-layer electrochemical flow-cell were used for the surface modification of the electrode. The flow-cell assembly consisted of a GC disk (about 144 mm^2 in area) as a working electrode, a silver-silver chloride reference electrode and a stainless-steel tube as an auxiliary electrode.

Prior to enzyme coating, the GC disk of both electrodes was polished with 1 μm diamond particles (BAS) and then 0.05 μm alumina particles (BAS), then washed with purified distilled water in an ultrasonic bath, and allowed to air-dry. The GC disks were then modified by cross-linking enzyme and gelatin using glutaraldehyde. The method was as follows. A 0.5 mg each of enzymes (tyrosinase, laccase, and peroxidase) and 10 μl of 5%(w/v) aqueous gelatin were added to 50 μl of 10 mM sodium

phosphate buffer (pH 6.5). A 10 μl aliquot of the resulting solution was spread over the GC disk of both electrodes and then they were exposed to glutaraldehyde vapor for 5 h in a desiccator. After that, the excess enzymes and residual aldehyde groups onto the enzyme membrane were removed by treating with glycine buffer (0.1 M, pH 7.5) for 12 h.

2.3. Apparatus and procedures

Cyclic voltammetric experiments were performed with a Fuso electrochemical system Model 312 connected to a XY recorder. All measurements were carried out in a three-electrode system with a bare GC electrode (5 mm in diameter) or enzyme-modified biosensor, an Ag/AgCl/KCl reference electrode, and a platinum wire auxiliary electrode, in a measuring cell thermostated at 25±0.2 ℃ containing 5 ml of a phosphate buffer.

The FIA system consisted of an Eicom GASTORR, a double-plunger μl pump (Eicom), an injector (Rheodyne 7735), an electrochemical detector (Eicom ECD-300) equipped with a potentiostat and a thin-layer flow-cell with an enzyme modified GC disk, and a SIC Chromatocorder. The flow-cell was connected to the outlet of the injector with an 100 cm length of PTFE coil (0.5 mm i.d.). A constant potential (-0.15 V *vs.* Ag/AgCl) was applied to the enzyme-based biosensor and the current was recorded. The 0.1 M sodium phosphate buffer (pH 5.5) was pumped as a working carrier solution, at a constant flow rate of 0.3 ml min^{-1}. Sample solutions (20 μl) were injected with a microsyringe.

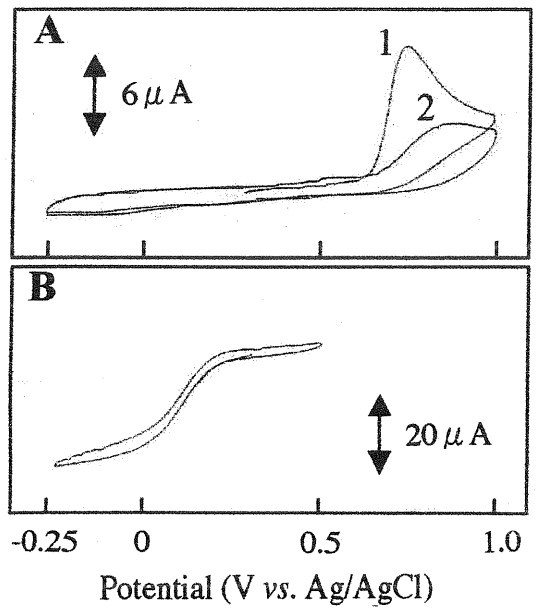

Fig. 1. Cyclic voltammograms of 0.05 mM 4-chlorophenol at bare glassy carbon electrode (A) and tyrosinase-based biosensor (B). The numbers 1 and 2 in upper recording: the first sweep and second sweep of potential toward the positive potential, respectively. The measurement were carried out in a 0.1 M sodium phosphate buffer (pH 5.5), with a scan rate of 50 mV s^{-1}.

3. Results and Discussion

3.1. Cyclic voltammograms of 4-chlorophenol (4-CP)

Cyclic voltammograms (CVs) of 4-CP were recorded at the bare GC electrode and the tyrosinase-based biosensors in a pH 5.5 sodium phosphate buffer and are shown in Fig. 1. At the bare GC electrode, 4-CP gave only single anodic-wave at a potential of about 700 mV and a small cathodic-wave on the sweep toward of negative potential. The anodic current markedly decreased at the second sweep of potential toward the positive potential. This means that the electropolymerization film is formed at the electrode surface during the electrooxidation as elucidated previously for phenol [19] and 1,2-diaminobenzene [20].

At the tyrosinase-based biosensor, however, 4-CP gave a well-defined cathodic wave at the potential around +0.1 V. The cathodic current was larger than the anodic current obtained at the bare GC electrode and no corresponding anodic-wave was observed. This means that the biocatalytic and electrochemical steps are effectively coupled in the tyrosinase-based biosensor to enhance the sensor sensitivity. In contrast the laccase-based biosensor and peroxidase-based biosensor (in the presence of 0.1 mM H_2O_2) gave a small cathodic-wave, respectively, compared to that obtained at the tyrosinase-based biosensor, although is not shown in Fig. 1.

The oxidation mechanisms of 4-CP catalyzed by tyrosinase can be presumed as shown in Fig. 2. The mechanism of monophenol hydroxylation and oxidation of produced diphenol to o-quinone by tyrosinase is completely elucidated by Wilcox *et al.* [21] and Sanchez-Ferrer *et al* [22]. According to the similar mechanisms, 4-CP is oxidized to 4-chlorobenzoquinone by tyrosinase which is reduced to 4-chlorocatechol at the electrode surface and at a suitable potential. The produced 4-chlorocatechol is oxidized back to the benzoquinone form by the tyrosinase-catalyzed reaction. As a result, an amplification of the current is obtained by such a substrate recycling reaction occurring between the enzyme membrane and the electrode.

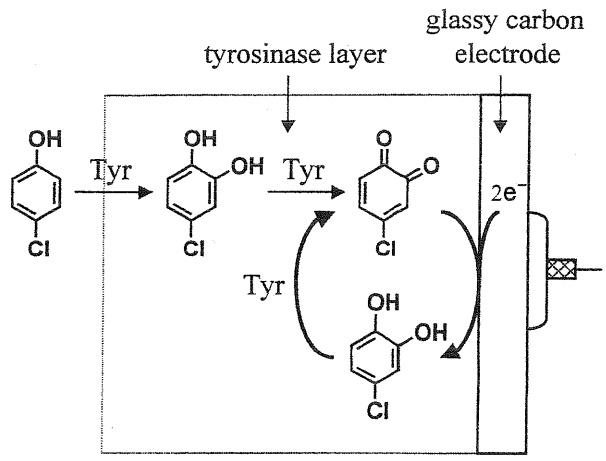

Fig. 2 Working principle for sensitive detection of 4-chlorophenol at tyrosinase (Tyr)-based biosensor.

3.2. Amperometric detection in a flow injection biosensor system

Three kinds of enzyme-based biosensor were used as an amperometric detector in a flow-injection system described in Experimental section. From a hydrodynamic voltammogram of 4-CP obtained at the tyrosinase-based biosensor, -0.15 V *vs.* Ag/AgCl was chosen as an optimum applied potential. The current response for 4-CP obtained at each of these biosensors is

compared in Table 1. Tyrosinase-based biosensor gave a extremely large signal to 4-CP compared with those obtained at laccase-based and peroxidase-based biosensors. Consequently, the tyrosinase-based biosensor was used throughout in this work.

Table 1 Compariaon of sensitivity of tyrosinase-based, laccase-based, and peroxidase-based biosensors to 0.1 mM 4-chlorophenol

Biosensor	peak current (nA)
Tyrosinase-based	12500
Laccase-based	73
Peroxidase-based	400

Carrier solution: 0.1 M sodium phosphate buffer (pH 5.5) for both the tyrosinase-based and laccase-based biosensors and the same buffer containing 0.1 mM H_2O_2 for the peroxidase-based biosensor. Carrier flow rate: 0.3 ml min^{-1}. Applied potential: -0.15 V *vs.* Ag/AgCl.

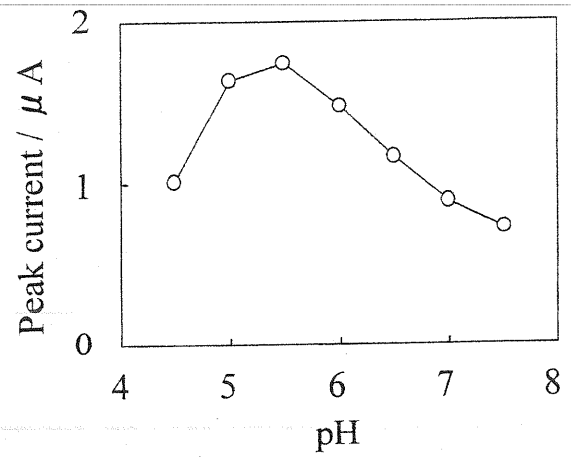

Fig. 3 Effect of pH of carrier buffer (0.1 M sodium phosphate) on the peak current of 10 μM 4-chlorophenol.

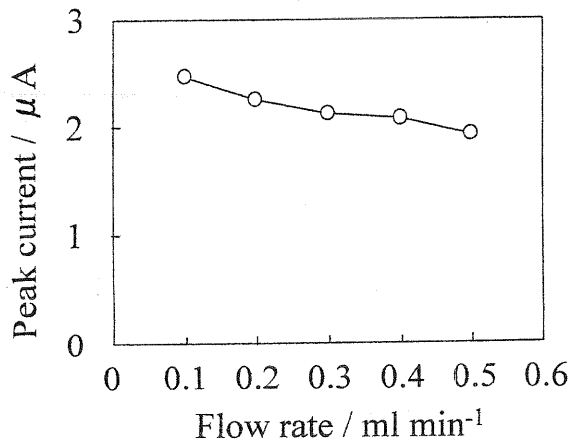

Fig. 4 Effect of the carrier flow rate on the peak current of 10 μM 4-chlorophenol. Carrier solution: 0.1 M, pH 5.5, sodium phosphate buffer. Applied potential: -0.15 V *vs.* Ag/AgCl.

Experiments were conducted to establish the optimum conditions for the use of the tyrosinase-based biosensor as an amperometric detector of 4-CP. Sodium phosphate buffers (0.1 M) at various pH values were tested as the carrier solution. As shown in Fig. 3, maximum response to 4-CP was observed at pH 5.5; at lower or higher pH values the current gradually decreased. Also, the carrier flow-rate is related to the residence time of the sample zone onto the tyrosinase-based biosensor. However, the signal current was almost independent on the carrier flow-rate over the range of 0.1 to 0.5 ml min^{-1}, as shown in Fig. 4. From these experimental results, a 0.1 M sodium phosphate buffer (pH 5.5) was selected as an optimum carrier and pumped at a flow rate of 0.3 ml min^{-1}. The biosensor was then poised at –0.15 V *vs.* Ag/AgCl.

Under these optimum flow conditions, the sensitivity for a variety of chlorophenols was evaluated from the peak currents obtained by 20 μl injection of 1 μM chlorophenols. The results are shown in Table 1. The tyrosinase-based biosensor responded sensitively to 4-CP, although gave a relatively small signal for each of 3-chlorophenol (3-CP) and 2,3-, 2,4-, and 3,4-dichlorophenols (2,3-DCP, 2,4-DCP, and 3,4-DCP) and no signal for trichlorophenols and pentachlorophenol.

Table 2 Substrate selectivity of the flow injection biosensor system for toxic chlorophenols

Chlorophenols (1 μM each)	Peak current (nA)	Relative response (%)
4-Chlorophenol	279	100
2-Chlorophenol	0.3	0.1
3-Chlorophenol	14.0	5.0
2,3-Dichlorophenol	5.8	2.1
2,4-Dichlorophenol	3.6	1.3
2,5-Dichlorophenol	n.d.	—
2,6-Dichlorophenol	n.d.	—
3,4-Dichlorophenol	8.5	3.0
3,5-Dichlorophenol	n.d.	—
2,3,4-Trichlorophenol	0.2	0.1
2,3,5-Trichlorophenol	n.d.	—
2,3,6-Trichlorophenol	n.d.	—
2,4,5-Trichlorophenol	n.d.	—
2,4,6-Trichlorophenol	n.d.	—
Pentachlorophenol	n.d.	—

n.d.: not detected.

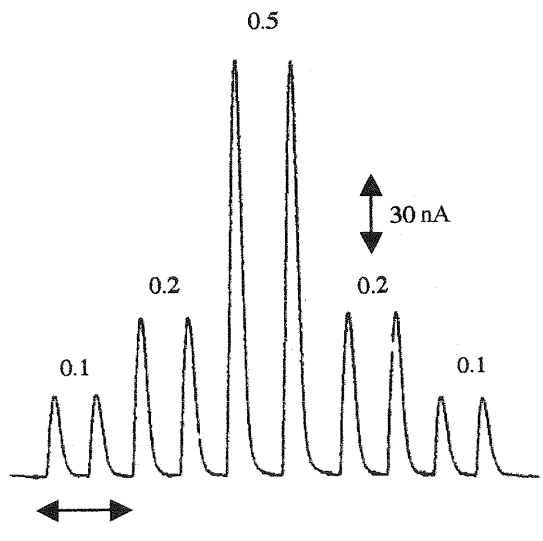

Fig. 5 Typical FIA signals for duplicate injections of standard solutions (0.1 – 0.5 μM) of 4-chlorophenol.

3.3 Precision and sensitivity

Figure 5 shows FIA signals of 4-CP. The peak-shaped signals without tailing were repeatedly obtained. The producibility of the measurements was tested by repeated injections (n=7) of the solution of 2×10^{-7} M 4-CP. The relative standard deviations was 0.98% for the flow injection biosensor system with the tyrosinase-based biosensor.

The calibration graphs for 4-CP was run using standard solutions of 4-CP over the concentration range between 5×10^{-9} and 1×10^{-5} M, which were injected in duplicate into the optimized flow-injection biosensor system. The strict linear relation was observed over a wide concentration range of 5×10^{-9} - 2×10^{-6} M for a 20 μl injection. The slope and y-intercept of the straight line with a correlation coefficient of 0.9985 were 279 nA μM^{-1} and 3.2 nA, respectively. The detection limit (S/N=3) was 3 nM (0.4 ppb). This detection limit was most sensitive compared to those obtained by the already published methods [1-11].

When the tyrosinase-based biosensor was stored at 4℃ in optimum phosphate buffer after the preparation, it retained most of its activity for four weeks. However, the operational stability was inferior to that of the storage stability; after the repetitive use of two weeks (5 h per day) the sensitivity to 4-CP decreased to about 50% of its original value, because of the inactivation of tyrosinase during the period of the use.

In conclusion, the flow injection system with the tyrosinase-based biosensor enabled the rapid and highly sensitive detection of 4-CP, based on the bioelectrocatalytic recycling occurring effectively at the tyrosinase / GC electrode interface of the biosensor. Some chlorophenols (3-CP, 2,3-DCP, 2,4-DCP, and 3,4-DCP) interfered with the measurement of 4-CP, but signals to such interferents were extremely small (< 5%) compared to that of 4-CP. The proposed biosensing FIA method will be useful for the trace analysis of 4-CP in the field of environmental and food analysis. Their analytical results will be also reported subsequently.

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