Int. J. Electrochem. Sci., 1(**2006**)55-61

International Journal of ELECTROCHEMICAL SCIENCE
www.electrochemsci.org

Tyrosinase Biosensor Based on Interdigitated Electrodes for Herbicides Determination

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Received: 9 May 2006 / *Accepted:* 24 May 2006 / *Published:* 27 May 2006

A conductometric tyrosinase biosensor has been developed to determine herbicides, alachlor, diazinon and carbaryl, in aqueous solutions. Tyrosinase from mushroom was immobilized on the sensitive part of interdigitated platinum planar electrodes by cross-linking with bovine serum albumin (BSA) in saturated glutaraldehyde (GA) vapor for 30 min. Herbicides inhibited tyrosinase, and the decrease of enzyme activity was used to determine these herbicides. Parameters influencing the performance of the systems were optimized to be used in the inhibition procedures. Detection limits for diazinon, alachlor and carbaryl were about 5.0×10^{-8}, 1.5×10^{-7} and 2.0×10^{-7} M, respectively. A relative standard deviation (RSD) (five times successive measurements) of the conductance responses is 5%. The 90% of the enzyme activity was still maintained after four weeks of storage at 4 ^{o}C in 5.0 mM phosphate buffer, pH 7.0.

Keywords: conductometric biosensor, enzyme, inhibitive, herbicide

1. INTRODUCTION

In recent years, herbicides and their metabolites, which show low environmental persistence but a high acute toxicity, have come into widespread use [1]. Their presence in surface and ground water poses a potential hazard to human health and there is a growing interest in their rapid and accurate determination. Standard procedures, based on liquid chromatography (LC) or gas chromatography (GC), are very reliable but there is a need for fast and innovative methods, especially in field and on-line applications. Electrochemical enzyme sensors are considered as an alternative to the conventional spectrometric techniques for pollutant determination due to their simplified sample

treatment, portable, economical, fast and sensitive analysis [2,3]. Biosensor can be easily combined with conventional electrochemical equipment and implement in the automated systems for the environmental monitoring or wastewater treatment control. Recent years, many amperometric biosensors based on the inhibition of the activity of tyrosinase enzymes have been used for the determination of triazine and phenylurea herbicides in the environment [4-7]. To our best knowledge, no previous work describing tyrosinase inhibition by these pollutants using conductometric sensors has been reported to date.

Conductometric sensors for biosensing devices have been introduced by Watson et al.[8]. The device consisted of an oxidized silicon substance with interdigitated platinum electrode pairs on one surface in a planar configuration. The principle of the detection is based on the fact that many biochemical reactions in solution produce changes in the electrical resistance. Conductance measurements involve the resistance determination of a sample solution between two parallel electrodes. For the direct assaying of many enzymes and their substrates, the conductometric biosensors present a number of advantages: (1) the planar conductometric electrodes are simple and relatively cheap which suits for miniaturisation and large scale production, and therefore promising for practical use, (2) they do not require a reference electrode, (3) the applied voltage can be sufficiently small to minimize substantially the sensor's power consumption, (4) large spectrum of analytes of different nature can be determined on the basis of various reactions and mechanisms.

This paper describes a conductometric biosensor for diazinon, alachlor and carbaryl determination in water solutions, based on their inhibitory effect on immobilized tyrosinase from mushroom and interdigitated thin-films planar electrodes. Experimental parameters such as the incubation time in pollutant solutions and the interference by others substances such as Cl^-, NO_3^-, SO_4^{2-} and heavy metals, have been investigated.

2. EXPERIMENTAL PART

2.1 Materials

Tyrosinase (T-7755; EC 1.14.18.1, 6680 U/mg) from mushroom, bovine serum albumin (BSA), catechol, glutaraldehyde (grade 2, 25% aqueous solution), diazinon, alachlor and carbaryl were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). All other reagents were of analytical grade and were used without any further treatment.

2.2. Sensor design

Conductometric transducers used, consisting of two identical pairs of platinum thin film electrodes, were fabricated at the institute of Chemo- and Biosensorics (Munster, Germany). The two pairs of Au (150nm thick) interdigitated electrodes were made by the lift-off process on the pyrex glass substrate. A 50 nm thick intermediate Ti layer was used to improve the adhesion of Au to the substrate. The central part of the sensor chip was closed by epoxy resin to define the electrode sensitive area. Both the digit width and interdigital distance were 10μm, and their length was about 1.0 mm. As a

result, the "sensitive " area of each electrode was about 1.0 mm^2 (Fig. 1). The internal generator of a Stanford Research System SR 830 lock-in amplifier (Sunnyvale, California, USA) was employed to generate a sinusoidal wave with a frequency of 100 kHz and a peak-to peak amplitude of 10 mV around a fixed potential of 0V to each pair of electrodes, forming a miniaturized conductance cell.

2.3 Enzyme immobilization

The biologically active membranes were prepared on the transducer surface by the cross-linking of enzyme with bovine serum albumin in saturated glutaraldehyde vapour [9]. A mixture containing 4% (w/w) tyrosinase, 5% bovine serum albumin, 10% glycerol in 20 mM phosphate buffer (pH 7.0) was deposited on the sensitive area of the sensor using a drop method, while another mixture of 10% (w/w) bovine serum albumin and 9% (w/w) glycerol in 20 mM buffer (pH 7.0) was deposited on the other electrode. The latter electrode was considered to be the reference sensor. The sensor chips were placed in a saturated glutaraldehyde vapour for 30 min followed by drying in air for 15 min at room temperature. Biosensors are prepared and then stored at 4 ^{o}C in the 5 mM phosphate buffer solution, pH 7.0, between the experiments.

2.4. Measurements

All measurements were performed in daylight at room temperature in an open vessel filled with the vigorously stirred 5.0 mM phosphate buffer solution, pH 7.0. Concentrations of substrates were adjusted by adding definite volumes of the stock solution of 200 mM catechol. The solutions of toxic substances were prepared in distilled water and used for enzyme inhibition in another vessel. The differential signal between the electrodes covered with the immobilized enzyme and those covered with the "blank" membrane was logged by the SR 830 lock-in amplifier and the steady-state response of the biosensors was plotted against the concentration of substrate.

The inhibition rate was determined by comparing the steady-state response of the conductometric biosensors before and after exposure to a sample solution containing the toxic compounds at the substrate concentration chosen.

3. RESULTS AND DISCUSSION

Tyrosinase is a binuclear copper containing metalloprotein which catalyses, in the presence of molecular oxygen, two different reactions: (1) the transformation of *o*-mono-phenols into catechols and (2) the oxidation of catechol to *o*-quinone. These reactions can be monitored by detection of conductivity variation. In this experiment, catechol was used as substrate for the determination of the herbicides.

Optimal characteristics of the tyrosinase biosensor were determined through the acquisition of calibration curves prior and after its incubation with a herbicide. The date in Fig. 2 presents the substrate concentration effect on the ratio (an index of residual activity of immobilized tyrosinase) between sensor responses prior and after incubation with diazinon. From Fig. 2, it can be seen that the

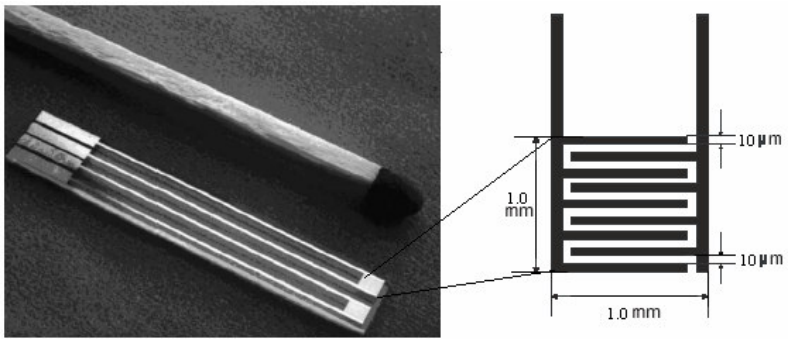

Figure1. Schematic diagram of the interdigitated thin-films planar electrode.

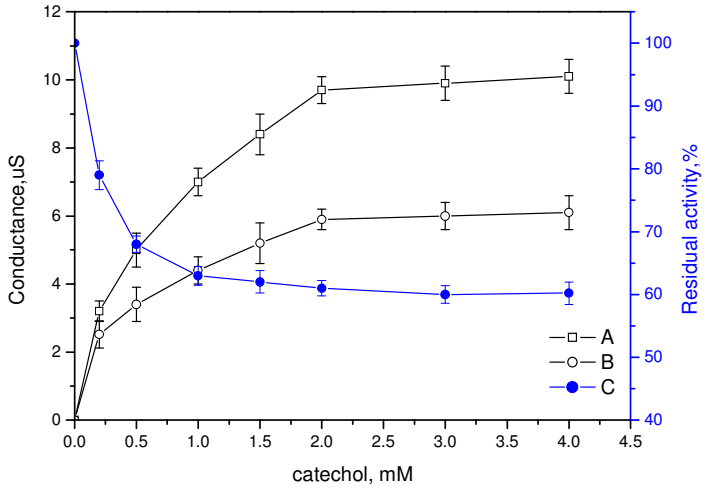

Figure2. Response dependece of the conductometric tyrosinase biosensor, before (A) and after (B) incubation with 1.0×10^{-5} M diazinon, on catechol concentration. Dependence of residual activity of immobilized tyrosinase on catechol concentrations (C). Measurements were conducted in triplicate in 5.0 mM phosphate buffer, pH 7.0, inhibition time, 30 min.

best sensitivity and accuracy of measurements is achieved at catechol concentrations higher than 2 mM. To select other optimal conditions for enzyme inhibition, the biosensor inhibition versus incubation time dependence was registered in the presence of different herbicides. Fig. 3 shows the effect of incubation time. The percentage of inhibition increased with incubation time for all herbicides. This is because the longer is the time, the more is the interaction between inhibitor and enzyme. However, a longer incubation time means a longer analysis time and a shorter lifetime of a biosensor. Therefore, incubation time was chosen as a compromise between the percentage of inhibition, analysis time and lifetime of the enzyme electrode. It can be seen in Fig. 3 that the optimal inhibition time for diazinon, alachlor and carbaryl was determined to be 30 min.

The calibration graph constructed by plotting the inhibition percentage of the enzyme activity against the concentration of different herbicides is shown in Fig.4. It is shown that the tyrosinase

activity is more inhibited by diazinon than by alachlor and carbaryl. Detection limits determined as the inhibitor's concentration giving a decrease of the substrate's signal equal to three times the blank value were: 5.0×10^{-8} M for diazinon, 1.5×10^{-7} M for alachlor and 2.0×10^{-7} M for carbaryl. In fact, the biosensor is not specific to individual herbicides but to a class of herbicides.

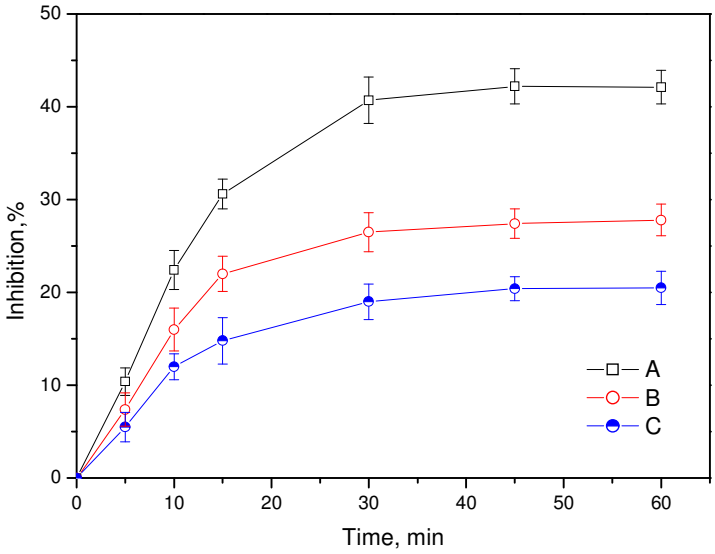

Figure 3. Dependence of the inhibition level of the tyrosinase bosensor on incubation time in solution of 1.0×10^{-5} M (A) diazinon, (B) alachlor, (C) carbaryl. Measurements were conducted in triplicate in 5.0 mM phosphate buffer, pH 7.0.

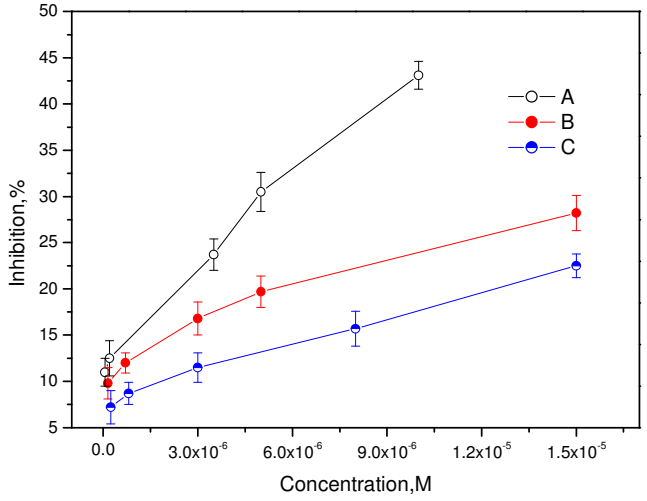

Figure. 4. Calibration curves of conductometric tyrosinase biosensor for (A) diazinon, (B) alachlor and (C) carbaryl. Measurements were conducted in triplicate in 5.0 mM phosphate buffer, pH 7.0, and incubation time of 30 min.

Therefore, the assay based on this biosensor is applicable for selective determination of a particular herbicide in certain geographic areas where the herbicide to detect is known in advance to be the only one. In any other case, this biosensor could be used as a rapid technique "early warning system" for measuring tyrosinase inhibitors present in the environment. For example, after preliminary results given by biosenors concerning environmental samples, only if an inhibition of tyrosinase activity occurs, validation and additional investigations using GC-MS or other classical methods could be performed.

In addition to herbicides tyrosinase can also be inhibited by inorganic inhibitors, such as Cl^-, NO_3^-, SO_4^{2-} [10-12]. Although these inorganics can inhibit tyrosinase, it was found that at the concentration of 1.0 mM, Cl^-, NO_3^-, SO_4^{2-} would provide a much less percentage of inhibition (3.5%) than that of diazinon at the concentration of 1.0×10^{-6} M. Therefore, these inorganics should not provide significant interference for the analysis of these herbicides. However, the biosensor may also serve as warming device for contamination by a high level of these inorganic inhibitors.

The influence of heavy metal ions such as copper, cadmium, zinc and lead ions has also been studied. These ions have been chosen both as being among the most relevant from the environmental point of view and because they are known to deactivate some enzymes. At 1.0 mM, the inhibition effect of copper was about one-tenth of diazinon at the concentration of 1.0×10^{-6} M while cadmium, zinc and lead did not show any significant inhibition. That is, although the heavy metals are at higher concentration level, diazinon, alachlor and carbaryl. should be the main inhibitors of tyrosinase.

The reproducibility of the tyrosinase conductometric biosensor was also investigated. Five successive measurements using the same biosensor were carried out at diazinon concentration level of 5.0×10^{-6} M. The relative standard deviation (RSD) of the conductance responses is 5%. The enzyme electrode was stored at 4 °C in 5.0 mM phosphate buffer (pH 7.0), and the sensitivity was analyzed every 3-4 days. The tyrosinase conductometric biosensor exhibited good stability with a relative of sensitivity of > 90% after 4 weeks of storage. This indicates a good long-term storage stability.

4. CONCLUSIONS

The developed conductometric tyrosinase biosensor was used for the determination of toxic compounds such as diazinon, alachlor and carbaryl. It showed a good performance for the determination of the herbicides with good sensitivity and detection limit. It was shown that the presence of Cl^-, NO_3^-, SO_4^{2-}, copper, lead and zinc, was unable to inhibit the tyrosinase in a studied concentration range.

The biosensor could certainly be used as a monitoring or a screening method for these herbicides. The major advantage of biosensor screening is that it allows the analysis of a large number of samples and only the positive ones need to be re-analyzed by chromatographic methods in order to determine the target pesticides responsible for the enzyme inhibition. It can provide a way to save time and costs, with a possibility of taking rapid decision about a local environmental problem.

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