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Evaluation of Pesticide and Heavy Metal Toxicity Using Immobilized Enzyme Alkaline Phosphatase with an Electrochemical Biosensor

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Abstract: An bioelectrochemical sensor or biosensor based on the inhibition of the enzyme Alkaline Phosphatase (ALP) has been investigated for the screening of several environmental toxicants. The biosensor was constructed by immobilizing ALP in a hybrid sol-gel/chitosan film that was deposited on the surface of a screen-printed carbon paste electrode (SPE). The inhibition was measured via the catalytic hydrolysis of ascorbic acid 2-phosphate (AA2P) by the enzyme to produce ascorbic acid. Oxidation of this product was monitored amperometrically and the current change was then related to ALP activity. Toxicity of herbicides (2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), insecticides (carbofuran and α-endosulfan) and heavy metals (Hg^{2+}, Cd^{2+}, Ag^{2+}, Zn^{2+} and Cu^{2+}) towards the biosensor were evaluated. Various degrees of inhibition of ALP occurred when the biosensor was exposed to herbicides and heavy metals. This resulted in a lower acid ascorbic production by the enzyme from the substrate, thus a decrease in the current response of the biosensor. The herbicides 2,4-D and 2,4,5-T showed the largest inhibition effect on ALP with linear response range of 1-60 μg L^{-1} (R^2 = 0.92). The maximum inhibitions caused by 2,4-D and 2,4,5-T were 46 and 30%, respectively. Heavy metals caused inhibition on ALP at the higher concentration range of mg L^{-1}. Thus, the biosensor may be useful for the screening of chlorophenoxyacetic acid herbicides even in the presence of other environmental toxicants.

Key words: Enzyme inhibition, electrochemical biosensor, herbicide, toxic metal

INTRODUCTION

The inhibition of enzymic reaction has been used very frequently to determine environmental pollutants like pesticides and heavy metals. Various enzymes such as acetyl cholinesterase (Chouteau *et al*., 2005; Carlo *et al*., 2004), alkaline phosphatase (Chouteau *et al*., 2005), urease (Rodriguez *et al*., 2004) and glucose oxidase (Malitesta and Guascito, 2005) have been reported for the construction of biosensors that used in the determination of pesticides or heavy metals.

Alkaline phosphatase, a non-specific phosphor monoesterase, is a dimeric metalloenzyme containing Zn^{2+} and Mg^{2+} ions coordinated to its active site. This enzyme has been use for indirect monitoring of pesticides or heavy metals that inhibit biocatalytic properties. Many studies have been published regarding the use of alkaline phosphatase in biosensor for the determination of pesticides or heavy metals, they included the amperometric (Mazzei *et al*., 2004), fluorimetric (Sanchez *et al*., 2004), colorimetric (Goh *et al*., 2005) and optical biosensors (Durrieu and Tran-Minh, 2002).

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The determination of pesticides and heavy metals is important due to their toxicity and persistency in the environment. These toxic substances have been shown to be responsible for many ecological problems and damages to human health (Argese *et al*., 2005). Several methods can be used for pesticides and heavy metals determination in water and soil samples, which mainly involved complicated instrumentation such as chromatography or atomic absorption spectrophotometers (Amarante *et al*., 2003; Vink and Poll, 1996). Despite their high sensitivity and accuracy, these instrumentations are expensive, time-consuming to operate and require skillful operator. For these reasons, biosensor method is more convenient because of low cost and portability especially for rapid screening of these pollutants.

Bioelectrochemical sensor, particularly amperometric biosensor is an analytical tool that utilizes immobilized biomolecules for the conversion of a specific target analyte into electrochemically detectable products. The electrochemically active product releases from the biochemical reaction between the recognition biomolecule and the analyte will be detected by an amperometric transducer. Recent development of biosensor techniques has seen the use of immobilized enzymes, DNA (Babkina and Ulakhovich, 2004), antibody (Blake *et al*., 2001) and microbe (Chouteau *et al*., 2005). The immobilization methods for these biomolecules are usually entrapment (Andreescu *et al*., 2002), adsorption (Bonnet *et al*., 2003) and covalent binding (Liu *et al*., 2005).

In this study, we have reported a biosensor based on the inhibition properties of the alkaline phosphatase enzyme for the detection of pesticides as well as heavy metals. Most of the biosensors reported for such purpose so far are complicated to fabricate, which also involved tedious operating procedures. Therefore, the objective of this study was to adopt a simple mean of fabricating such a biosensor by immobilizing the alkaline phosphatase (ALP) enzyme in a sol gel/chitosan membrane and then investigate the inhibition of the enzyme by several herbicides, insecticides and heavy metals via electrochemical transduction. The membrane could be directly prepared via a one-step procedure without any complicated chemical immobilization and multilayer assembly of ALP (Chen *et al*., 1995). An electrochemical biosensor was constructed based on the immobilization of ALP in the sol gel/chitosan membrane. Both sol-gel and chitosan can be used as immobilization matrixes for biosensor because of their inertness and biocompatible properties (Miao and Tan, 2001).

MATERIALS AND METHODS

Reagents

Enzyme alkaline phosphatase (EC 3.1.3.1) from bovine (Sigma), tris-HCl buffer 0.1 M, pH 8.5; ascorbic acid 2-phosphate (AA2P) (Sigma) was prepared in distill water to give a final concentration of 0.1 M; 2, 4-dichlorophenoxyacetic acid (2, 4-D), 2, 4, 5-Trichlorophenoxyacetic acid (2, 4, 5-T), carbofuran and α-endosulfan were from Sigma. All metal ion solution $HgNO_3$ (Merck), $AgNO_3$ (Ajax Chemical), $CdCl_2$ (General Purpose Reagent), $ZnNO_3$ and $CuSO_4$ (BDH Laboratory Supplies) were prepared in distilled and deionized water. Tetraehoxythosilane (TEOS) was from Fluka; hydrochloric acid and glacial acetic acid from Merck. Chitosan and $MgCl_2$ were from Sigma.

Procedures

This research study was conducted at the laboratory of Chemical Sensor and Biosensor Research Group, Universiti Kebangsaan Malaysia at the end of the year 2006. The research was performed over a period of 3-4 months.

Preparation of Electrochemical Biosensor

The hybrid sol-gel/chitosan membrane was prepared by adding 2 mL of TEOS, 3 mL of deionized water and 0.10 mL of 1 M HCl in a glass vial and stirred for 1 h. The 1% chitosan solution was

prepared in 1% acetic acid and stirred to dissolve overnight. The hybrid of sol-gel/chitosan was prepared by mixing both sol-gel and chitosan solution with the ratio of 9:1 (w/w). 5 microliter of hybrid sol-gel/chitosan solution which containing ALP (2 unit m L^{-1}) was deposited on the surface of SPE and allowed to dry. A thin layer of film containing entrapped ALP was formed. These electrodes were then used in the investigation of the behavior of the immobilized ALP via electrochemical transduction.

Electrochemical Measurements

The amperometric measurements were performed using Autolab PGSTAT 12 Potentialstat/Galvanostat. The working electrode was a screen-printed carbon electrode (SPE), which is designed by the Chem Biosensor Research Group of the National University of Malaysia and manufactured by Scrint Print Co, Malaysia. The amperometric measurements were performed at 0.6 V versus Ag/AgCl as the reference electrode and a platinum electrode was used as an auxiliary electrode. A magnetic stirrer was used to stir the solution during amperometric measurements. All experiments were performed in 0.1 M Tris-HCl buffer, pH 8.5 containing 1 mM $MgCl_2$.

Inhibition Studies of ALP Enzyme by Toxicants

The inhibition of the ALP enzyme was carried out by immersing the SPE coated with enzyme film into the pesticide or metal ion standard solution of 1-200 μg L^{-1} for 15 min incubation. The current Io of the biosensor was measured before the incubation. After the incubation, the current I_A of the biosensor was measured again.

Therefore, the percentage of inhibition (% I) can be calculated based on current measured before and after exposure to the toxicants as follows:

$$\text{Inhibition (\%I)} = \frac{I_0 - I_A}{I_0} \times 100$$

RESULTS AND DISCUSSION

Inhibition of ALP by Pesticides

The enzyme ALP can catalyse the hydrolysis of ascorbic acid 2-phosphatase (AA2P) to ascorbic acid. Oxidation of ascorbic acid at the electrode lead to current changes and this is an indirect measurement of ALP activity (Fig. 1). Whenever a sample that contains herbicide or metal ion is added to the coupled enzyme reaction and inhibited the biocatalytic properties of biosensor, the ascorbic acid production will be reduced. Thus, ascorbic acid oxidation will occur at a lower rate. By comparing the oxidation rate for acid ascorbic in an uninhibited condition, the presence of herbicide and metal ion in a sample can be determined.

The inhibition behaviour of ALP enzymic activity can be considered as an efficient signal regarding the presence of pollutants in a sample. For 2,4-D and 2,4,5-T, both showed similar inhibition profiles (Fig. 2) and the maximum inhibition occurred at 100 μg L^{-1} even though the concentration studied was 1-200 μg L^{-1}. The maximum inhibition was 46 and 30%, respectively for 2,4-D and 2,4,5-T. However, the inhibition could not achieved 100% because these herbicides are rather weak inhibitor for ALP and longer incubation time than employed here (15 min) might be needed to achieve higher level of inhibition. The linear response range of inhibition of ALP by both herbicides is the same, i.e., 1-60 μg L^{-1} (R^2 = 0.928 and 0.913 for 2, 4-D and 2, 4, 5-T, respectively). The limit of detection (LOD) of the biosensor for 2, 4-D and 2, 4, 5-T are 0.5 and 0.8 μg L^{-1}, respectively with the response time of less than 5 min. The reproducibility of the biosensor is within 1.0-6.0% Relative Standard Deviation (RSD). The World Health Organization (WHO) recommended that the maximum safety level of 2, 4-D and 2, 4, 5-T should not exceed 30 μg L^{-1} in drinking water. Therefore, the biosensor developed here can be used to screen the presence of herbicides because of its low detection

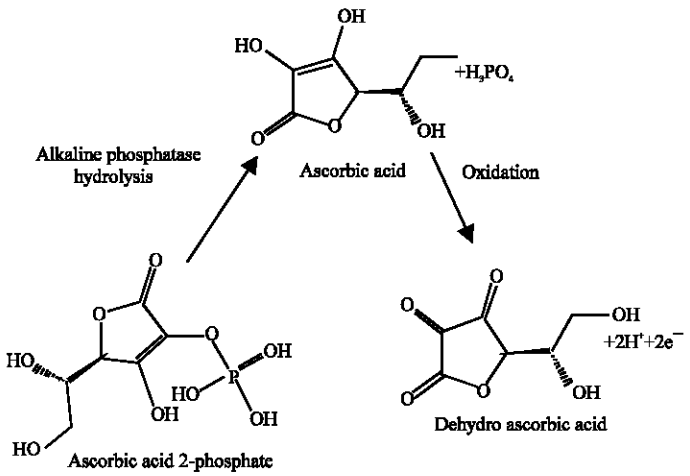

Fig 1: The hydrolysis of ascorbic acid 2-phosphate (AA2P) by enzyme ALP to form ascorbic acid. Oxidation of ascorbic acid at the carbon paste SPE to produce dehydro ascorbic acid with the release of electrons that generate a measurable amount of current

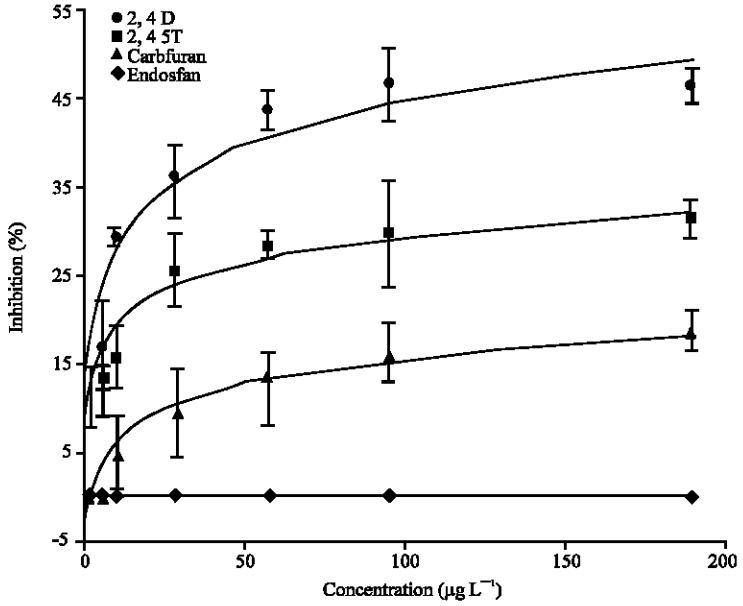

Fig. 2: The inhibition profiles of the alkaline phosphatase in the presence of each pesticide. (Experimental conditions: Substrate AA2P 80 µM, Tris-HCl buffer 0.1 M, pH 8.5, applied potential 0.6 V versus Ag/AgCl)

limit and suitable linear response range. On the other hand, for other pesticides, a relatively lower inhibition was observed for the insecticide carbofuran and no response for α-endosulfan (Fig. 2).

Besides for the detection of herbicides, alkaline phosphatase has been used for biosensor in the detection of organophosphorus pesticides (e.g., paraoxon), where, it was first conjugated with

streptavidin before immobilized in a polymer matrix on glass surface (Ayyagari *et al*., 1995). The synthesis of the conjugated copolymer, poly (3-undecylthiophene-co-3-thiophenecarboxyldehyde-biotin-LC-hydrozone) was complicated and the level of pesticides detected was similar to the sol-gel/chitosan based membrane biosensor reported here.

The detection limits for 2, 4-D and 2, 4, 5-T using an electrochemical immunosensor were reported to be 40 μg L^{-1} of 2, 4-D and 50 μg L^{-1} of 2, 4, 5-T in both water and serum (Yulaev *et al*., 1996). The selectivity of this immunosensor to the herbicides was good because of the very specific binding behaviour from the conformation of the active site in the antibody molecule. However, its sensitivity was lower when compared to this study considering much effort has been made on the fabrication of this immunosensor.

Inhibition of ALP by Heavy Metals

Beside pesticides, the activity of ALP can be inhibited by some heavy metals (Sanchez *et al*., 2003). The profiles of inhibition of ALP by various heavy metals (Hg^{2+}, Cd^{2+}, Ag^{2+}, Zn^{2+} and Cu^{2+}) are depicted in Fig. 3. The inhibition effect of Hg^{2+} on ALP was the highest among the metal ions studied, i.e., 40 mg L^{-1} of Hg^{2+} caused an approximately 60% inhibition. The inhibition for other heavy metals was 42% for Cu^{2+}; 37% for Cd^{2+}; 30% for Zn^{2+} and 26% for Ag^{+} at 40 mg L^{-1} of metal concentration. Thus, the trend of response is Hg^{2+}>Cu^{2+} Cd^{2+} Zn^{2+} Ag^{+}. The metal ions Hg^{2+} and Cu^{2+} exhibited stronger inhibition on ALP because the type of inhibition involved is non-competitive (Chen *et al*., 2000) and this leads to direct inhibition of ALP without undergoing competition with the enzyme substrate.

Chouteau *et al*. (2005) have reported a conductometric electrode with immobilized microalgae *Chlorella vulgaris* as a bioreceptor for heavy metals (Cd^{2+} and Zn^{2+}) detection. The inhibition of ALP in the membrane of *C. vulgaris* has demonstrated a lower LOD when compared to the amperometric biosensor reported here. This is due to the higher amount of ALP from a higher concentration of algae

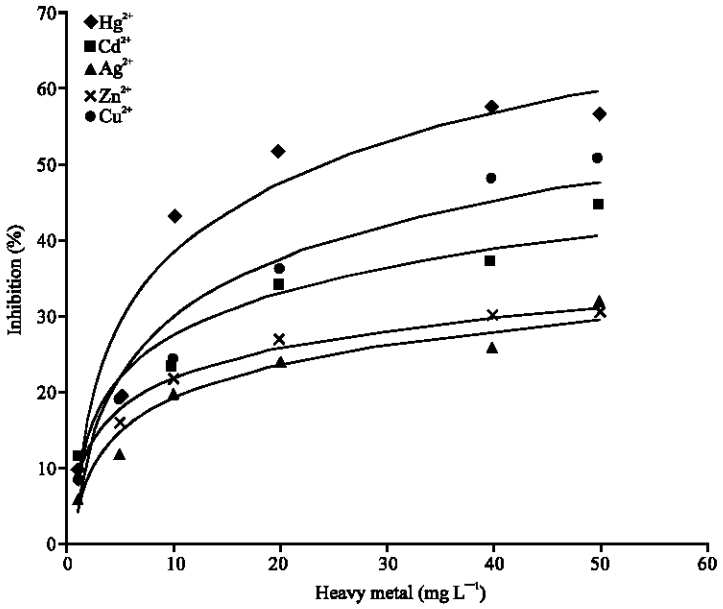

Fig. 3: The inhibition profiles of ALP by several metal ions. Experimental conditions are similar to Fig. 2

Table 1: The concentrations of various toxicants required to cause 30% inhibition to the ALP as measured by the biosensor

Toxicant	Concentration at 30% inhibition ($I_{0.3}$) ($\mu g\ L^{-1}$)
2,4-D	10
2,4,5-T	100
Carbofuran	>200
Hg^{2+}	5400
Cu^{2+}	10200
Cd^{2+}	13800
Zn^{2+}	41700
Ag^{+}	50000

used ($4\text{-}5\times10^{7}$ cells m L^{-1}) when compared with 2 units m L^{-1} of ALP employed on screen printed electrode reported here. As the enzyme concentration increases, the toxicants can be detected at lower detection limit. Besides, the algae biosensor was pre-incubated for 30-60 min in test solution, which is longer than in this study.

At 30% inhibition level that normally considers suitable for inhibition studies, 2,4-D and 2,4,5-T herbicides showed the highest sensitivity (Table 1). The biosensor is less sensitive to heavy metals and to attain 30% inhibition, the concentrations of heavy metals need to be 1000-5000 times higher than that of herbicides. This demonstrates that the biosensor developed here has good selectivity towards the chlorophenoxyacetic acid herbicides when compared with other pesticides and toxic metals.

CONCLUSION

This study has proved that a toxicity biosensor could be constructed from the immobilization of alkaline phosphatase enzyme in a hybrid sol-gel/chitosan membrane and an electrochemical biosensor could be used to measure the toxicity caused by several toxicants. The biosensor showed inhibition by herbicides, carbofuran insecticide and toxic metals with various degrees of inhibitory response. It is most inhibited by the chlorophenoxyacetic acid herbicides and thus the biosensor demonstrated good sensitivity and selectivity for these compounds. The simple design and direct fabrication procedure of this biosensor are useful for a disposable device that can detect broad spectrum toxicity of water.

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