Alkaline phosphatase inhibition based electrochemical sensors for the detection of pesticides

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Abstract

This article presents a bioelectrochemical system for the determination of pesticides by enzyme inhibition data. Measurements are performed by electrochemically monitoring the inhibition of the catalytic activity of the enzyme alkaline phosphatase (ALP) either in the presence or in the absence of pesticides, and particularly of Malathion and of 2,4-dichlorophenoxyacetic acid (2,4-D), representatives of the general classes of organophosphorous and organochlorinated agents. ALP catalytic activity was determined by using two different analytical configurations: (a) an amperometric ALP based biosensors using 3-indoxyl phosphate as the enzyme substrate; (b) a system allowing the voltammetric determination of the electroactive products of the ALP catalyzed reactions by using two different substrates: phenyl phosphate (PP) and ascorbate-2-phosphate (A-2-P). Studies of cyclic voltammetry and amperometry were performed first to define the optimal experimental conditions of the electrochemical measurements. For the optimal experimental conditions (Tris–0.1 M HCl aqueous buffer solution at pH 8.0, incubation time of 30–60 min) a detection limit of 0.5–6 lgl0 is obtained, with a response, which is linear over 1–2 decades of concentration.

Keywords: Enzymatic inhibition; Amperometry; Voltammetry; 2,4-Dichlorophenoxyacetic acid; Malathion; Alkaline phosphatase

1. Introduction

The wide use of pesticides and herbicides in agriculture to preserve crops from pests, as well as their high intrinsic toxicity, associated, in many instances, with a remarkable persistence in the environment, represents a potential danger for the health of ecosystems. The control of their concentration, in different matrices (primarily waters and soil), is a key component of any strategy of environmental management and control.

A wide number of analytical methods, based on the most commonly employed physico-chemical techniques for the identification of organic compounds (UV, IR, HPLC, GC, MS or various combinations of these) [1–5], is available to detect and determine pesticides quantitatively in different matrices; nevertheless, none of the above mentioned techniques is effectively used “on the spot” to monitor the levels of pesticides in risk areas continuously: in this direction, the use of alternative analytical methods, such as those based on bioelectrodes...
would be very welcome, since they could represent one of the most practical and inexpensive analytical devices, especially for the preliminary screening of huge numbers of samples.

We have previously published several inhibition based biosensors for the determination of different environmental pollutants, including marine toxins and pesticides [17–23] by enzyme inhibition studies; we are now proposing a further development of our approach, presenting two biodevices for the determination of pesticides based on the electrochemical monitoring of the catalytic activity and inhibition of alkaline phosphatase (ALP) by direct amperometric determination of the enzyme catalyzed reaction products.

Studies of cyclic voltammetry and amperometry were performed first to define the optimal experimental conditions of the electrochemical measurements. Details are given on the practical construction of this biosensor and on its application to standard solutions of pesticides.

2. Experimental

2.1. Reagents and solutions

The enzyme ALP (EC 3.1.3.1 from Bovine Liver), 3-indoxyl phosphate (3-IP), phenyl phosphate (PP), ascorbate 2 phosphate (A-2-P), 2,4-D and Malathion were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were analytical grade. Twice distilled water was used for the preparation of all solutions.

Biodyne transfer membranes, (nylon 6.6 membrane, 200 μm thickness, pore size 0.45 μm) with carboxylic groups on the surface, were supplied by Pall Italia s.r.l. (Milano, Italy). Cellulose acetate H₂O₂-selective membranes (100 μm thickness, M_w cutoff ≥ 100) were prepared according to a recently described procedure [24]. Cellulose acetate dialysis membranes (0.0254 mm thick; M_w cutoff = 12,000 D), were supplied by Sigma Chemical Co. (St. Louis, MO, USA). The polyazetidine prepolymer (PAP) solution (Hercules Polycup 172, 12% solids in water), used for the physico-chemical immobilization of the enzymes, was obtained from Hercules Inc. (Wilmington, Del. USA).

2.2. Electrochemical measurements

(a) Amperometric biosensor: The proposed biosensor is based on electrochemical measurement of the activity of the enzyme alkaline phosphatase (ALP), catalyzing the first reaction of the sequence shown in Fig. 1. ALP is coupled to an amperometric hydrogen peroxide sensitive electrode, so that the measured changes of current intensities allow the determination of H₂O₂, which is proportional to the concentration of 3-IP in the sample. The determination of pesticides is carried out by monitoring the inhibition of the catalytic activity of ALP by the amperometric biosensor, which leads to a reduction of the H₂O₂ produced at the electrode.

2.3. Preparation of the enzymatic electrode

The biosensor was prepared by immobilizing an adequate amount of ALP on a nylon 6.6 membrane with carboxylic groups on the surface, by means of polyazetidine. ALP and PAP (1 mg ALP/10 μl PAP) were spread uniformly on a disk of the membrane (0.8 cm diameter; density of enzyme ≈ 0.135 mg/cm²). The enzymatic membrane was left for 24 h at room temperature, washed with 0.1 M, pH 8.0 glycine buffer and then stored in a dry state at 0 °C.

2.4. Assembly of the sensor

The sensor was assembled by placing on the platinum surface of a H₂O₂ electrode (Universal Sensors Inc., New Orleans, LA, USA) a sequence of three different membranes, in the following order: (i) a cellulose acetate membrane (to eliminate possible interference from other electroactive chemical species); (ii) the GOD-nylon membrane; (iii) the ALP-dialysis membrane.

A rubber O-ring was used to fix the above mentioned three successive layers on the tip of the H₂O₂ electrode. A schematic representation of the bienzymatic biosensor is presented in Fig. 2.

2.5. Storage of the sensor

For short-term storage (up to 8 h), the biosensor was kept in a Tris–0.1 M HCl buffer solution at pH 8.0. For longer-term storage the enzymatic membranes were removed and kept dried at −15 °C.
2.6. Amperometric measurements

Amperometric measurements were carried out by connecting the biosensor to an amperometric detector (ABD, Universal Sensors Inc., New Orleans, LA, USA). A constant potential of +650 mV was applied between the platinum anode and the Ag\textsubscript{j}AgCl cathode of the hydrogen peroxide electrode. The electrode jacket was filled with an internal filling solution of phosphate buffer and KCl, both 0.1 M, pH 6.6.

2.7. Experiments with the enzymatic biosensor

To quantify the degree of inhibition of pesticides towards ALP we performed a set of experiments with the ALP based enzymatic biosensor. Experiments were carried out in a glass cell, thermostated at 37 °C by forced water circulation, ensuring a uniform magnetic stirring, at a constant rate, throughout the assay, in 2.5 ml of Tris–0.1 M HCl buffer solution at pH 8.0, containing 0.2 mM MgCl\textsubscript{2} as the ALP cofactor. The determination of pesticides was performed by dipping the ALP biosensor in Tris–0.1 M HCl buffer solution at pH 8.0, at 37 °C, and adding 3-indoxyl phosphate at a final concentration of 0.4 mM: this value was chosen since it represents the best compromise between the optimal bioelectrode responses (it lies in the middle of the linearity range for the 3-IP biosensor) and the optimal concentration ratio of 3-IP/inhibitor to highlight the inhibition of the catalytic activity of ALP quantitatively. After stabilization of the current, increasing quantities of the solution containing pesticide were added under constant magnetic stirring, and the decrease of the current, proportional to the decrease of the hydrogen peroxide production following ALP inhibition, was followed for 30 min. In this way a calibration curve reporting the decrease of current as a function of the concentration of added inhibitor could easily be derived.

(b) Voltammetric method: We are here proposing a simpler system, based on the monitoring of the catalytic activity of alkaline phosphatase (ALP) by the direct amperometric determination of the enzyme catalyzed reaction products. Phenyl phosphate (PP) and ascorbate-2-phosphate (A-2-P) were chosen as substrates of the ALP catalyzed reaction:

\[
\text{Ascorbic acid}_2 \text{phosphate} \leftrightarrow \text{Ascorbic acid} + \text{H}_3\text{PO}_4
\]

(1)

\[
\text{Phenyl phosphate} \leftrightarrow \text{Phenol} + \text{H}_3\text{PO}_4
\]

(2)

2.8. Apparatus

All electrochemical measurements were performed by an Autolab PG stat 10 potentiostat (Eco Chemie B.V., Utrecht, The Netherlands) interfaced to a personal computer and controlled by its dedicated software (Autolab GPES version 4.7 for Windows). Measurements were carried out at 37 °C in a BAS Unicell electrochemical flow cell (BioAnalytical Systems Inc., West Lafayette, Indiana, USA) with a three electrode configuration: the working electrode was a glassy carbon with a surface diameter of 3 mm, used together with a platinum counter electrode and an Ag\textsubscript{j}AgCl/KCl reference electrode. A peristaltic pump (Gilson Minipuls 3, Gilson Italia Srl, Cinisello Balsamo, Milano, Italy) was used to ensure the substrate intake.

2.8.1. Cyclic voltammetry

Flow analyses were performed in order to evaluate the method proposed here. Comparative studies of cyclic voltammetry were performed, for the monitoring of ALP activity and inhibition in the presence of the different substrates A-2-P and PP. Scanning voltammograms were obtained over the potential range of 0–1.0 V (scan rate: 20 mV s\textsuperscript{-1}) with respect to the Ag\textsubscript{j}AgCl reference electrode, in the presence of the substrates for different levels of ALP.

For the amperometric measurements, the optimal potential values were found to be +850 and +400 mV for the products of the ALP enzymatic reaction for the substrates employed, respectively, ascorbic acid (reaction (1)) and phenol (reaction (2)).

2.8.2. Amperometric measurements

In order to evaluate the inhibition effect of 2,4-D and Malathion on the catalytic activity of ALP we
performed a set of experiments with the enzyme free in solution. Experiments were carried out at 37 °C in a flow cell, at a constant flow rate of 0.5 ml/min. In the measurements we used a Tris–0.1 M HCl buffer solution at pH 8.0, containing ALP (final concentration: 1.0 mU l⁻¹) and its cofactor 0.2 mM MgCl₂. The determination of pesticides was performed by adding either PP or A-2-P to a final concentration of 5 μM: this value was chosen since it represents the best compromise between the optimal electrochemical device responses and the optimal concentration ratio of substrate/inhibitor to highlight the inhibition of the catalytic activity of ALP quantitatively. The same experiments were performed in the presence of increasing concentrations of pesticides, added from a stock solution prepared daily. The decrease of the current observed was proportional to the decrease of the phenol and ascorbate production following ALP inhibition. Calibration curves were obtained reporting the decrease of current intensity at a fixed pesticide incubation time (30 and 60 min) as a function of the concentration of pesticide added.

3. Results

(a) Amperometric biosensor: Table 1 summarizes the main electroanalytical features of the proposed method of the enzymatic biosensor as evaluated on standard solutions of 2,4-D and Malathion.

Fig. 3 shows the trend of the amperometric signal recorded during a typical inhibition experiment, followed by the enzymatic ALP biosensor. The first arrow refers to the addition of 3-IP, to a final concentration of 0.4 mM, the second arrow to the addition of pesticide. As can be seen, the value of the current intensity increases after the addition of 3-IP and decreases after the addition of the pesticide, thus confirming the inhibition power of these substances towards the catalytic activity of ALP.

(b) Voltammetric method: Table 2 reports the main electroanalytical features obtained in the analysis of a standard solution of the two substrates, A-2-P and PP, with the method proposed in this paper. The main electroanalytical properties obtained by monitoring the ALP activity inhibition for different incubation times (30 and 60 min) in the presence of 2,4-D standard solutions, with either one of the two enzyme substrate, are reported in Table 3. Table 4 shows the results obtained in the same inhibition experiments performed in the presence of known concentrations of Malathion.

4. Discussion

Kinetic assays involving inhibition of specific enzymatic systems have been applied extensively as analytical methods for the detection of food and environmental contaminants, primarily among them, pesticides, herbicides and heavy metals. A particular form of enzymatic inhibition assay is represented by enzymatic inhibition bioelectrodes, whose flexibility

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Table 1
Analytical characterization of the ALP-based biosensor for the determination of 2,4-D and Malathion

<table>
<thead>
<tr>
<th>Pesticide</th>
<th>2,4-D</th>
<th>Malathion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature of analysis (°C)</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>pH</td>
<td>8.0</td>
<td>8.0</td>
</tr>
<tr>
<td>Buffer</td>
<td>Tris–0.1 M HCl + 1 mM MgCl₂</td>
<td>Tris–0.1 M HCl + 1 mM MgCl₂</td>
</tr>
<tr>
<td>Linearity range (μg l⁻¹)</td>
<td>1.5–60.0</td>
<td>0.2–45.0</td>
</tr>
<tr>
<td>Equation of the calibration graph: Y = ΔI (nA); X = substrate concentration (μg l⁻¹)</td>
<td>Y = 1.2 + 20.5X</td>
<td>Y = 0.3 + 30.9X</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.9990</td>
<td>0.9995</td>
</tr>
<tr>
<td>Lower detection limit (μg l⁻¹)</td>
<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td>Life time as number of assays</td>
<td>60</td>
<td>80</td>
</tr>
<tr>
<td>Repeatability of the measurements</td>
<td>1.0</td>
<td>1.2</td>
</tr>
<tr>
<td>(as pooled standard deviation in the linearity range)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
can ensure the analysis of a huge population of samples at very reduced costs.

An “ideal” inhibition biosensor should, in principle, ensure the rapid detection of all contaminants endowed with the same biological effect, without the need for an extensive sample pretreatment. In this context, the use of an enzyme as the biological component of the biosensor ensures, in principle, the detection of all compounds affecting the enzyme catalytic activity.

From the experimental results presented here, the proposed inhibition based electrochemical method appears to be a promising tool for the rapid determination of 2,4-D and Malathion, and, as outlined above, also of related compounds capable of inhibiting ALP (i.e., marine toxins and other environmental pollutants).

The specific advantage towards other bioelectrochemical systems is primarily represented by the fact that, in the work presented here, only one enzyme is required for the transduction of the analytical signal, which is indeed dependent on the inhibition of the catalytic activity of ALP. In other words, the proposed bio-devices are simplified with respect to more complex

### Table 2
Analytical characterization of the ALP-based bioelectrochemical system for the determination of ascorbate-2-phosphate and phenylphosphate, in the presence of 1.0 U/ml of enzyme

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Ascorbate 2 phosphate</th>
<th>Phenyl phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature of analysis (°C)</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>pH</td>
<td>8.0</td>
<td>8.0</td>
</tr>
<tr>
<td>Buffer</td>
<td>Tris-0.1 M HCl + 0.2 mM MgCl₂</td>
<td>Tris-0.1 M HCl + 0.2 mM MgCl₂</td>
</tr>
<tr>
<td>Linearity range (μM)</td>
<td>0.5–80.0</td>
<td>0.5–40.0</td>
</tr>
<tr>
<td>Equation of the calibration graph: ( Y = \Delta I ) (nA); ( X ) = substrate concentration (μM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.9993</td>
<td>0.9990</td>
</tr>
<tr>
<td>Lower detection limit (μM)</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Repeatability of the measurements</td>
<td>1.2</td>
<td>1.6</td>
</tr>
</tbody>
</table>

(As pooled standard deviation in the linearity range)

### Table 3
Analytical characterization of the ALP-based bioelectrochemical system for the determination of 2,4-D using A-2-P and PP as substrate, in the presence of 1.0 U/ml of enzyme

<table>
<thead>
<tr>
<th>Substrate</th>
<th>A-2-P</th>
<th>A-2-P</th>
<th>PP</th>
<th>PP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potential applied (mV vs. Ag/AgCl)</td>
<td>400</td>
<td>400</td>
<td>850</td>
<td>850</td>
</tr>
<tr>
<td>Incubation time (min)</td>
<td>30</td>
<td>60</td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td>Linearity range (μg l⁻¹)</td>
<td>5.0–40.0</td>
<td>2.0–40.0</td>
<td>8.0–60.0</td>
<td>4.0–75.0</td>
</tr>
<tr>
<td>Equation of the calibration graph: ( Y = \Delta I ) (nA); ( X = 2,4-D ) concentration (μg l⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.9980</td>
<td>0.9990</td>
<td>0.9992</td>
<td>0.9984</td>
</tr>
<tr>
<td>Lower detection limit (μg l⁻¹)</td>
<td>2.0</td>
<td>1.0</td>
<td>6.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Repeatability of the measurements</td>
<td>2.3</td>
<td>1.8</td>
<td>2.0</td>
<td>1.9</td>
</tr>
</tbody>
</table>

(As pooled standard deviation in the linearity range)

All the measurements were performed at 37 °C in Tris-0.1 M HCl buffer solution at pH 8.0, containing 0.2 mM MgCl₂ as the ALP cofactor.

### Table 4
Analytical characterization of the ALP-based bioelectrochemical system for the determination of Malathion using A-2-P and PP as substrate, in the presence of 1.0 U/ml of enzyme

<table>
<thead>
<tr>
<th>Substrate</th>
<th>A-2-P</th>
<th>A-2-P</th>
<th>PP</th>
<th>PP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potential applied (mV vs. Ag/AgCl)</td>
<td>400</td>
<td>400</td>
<td>850</td>
<td>850</td>
</tr>
<tr>
<td>Incubation time (min)</td>
<td>30</td>
<td>60</td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td>Linearity range (μg l⁻¹)</td>
<td>2.0–35.0</td>
<td>0.5–45.0</td>
<td>4.0–48.0</td>
<td>2.5–65.0</td>
</tr>
<tr>
<td>Equation of the calibration graph: ( Y = \Delta I ) (nA); ( X = \text{Malathion} ) concentration (μg l⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.9990</td>
<td>0.9988</td>
<td>0.9992</td>
<td>0.9990</td>
</tr>
<tr>
<td>Lower detection limit (μg l⁻¹)</td>
<td>1.0</td>
<td>0.25</td>
<td>2.0</td>
<td>1.25</td>
</tr>
<tr>
<td>Repeatability of the measurements</td>
<td>1.7</td>
<td>1.4</td>
<td>1.4</td>
<td>1.8</td>
</tr>
</tbody>
</table>

(As pooled standard deviation in the linearity range)

All the measurements were performed at 37 °C in Tris-0.1 M HCl buffer solution at pH 8.0, containing 0.2 mM MgCl₂ as the ALP cofactor.
bioelectrochemical arrangements (i.e., bioenzymatic systems, with or without chemical mediators) yet maintain a satisfactory analytical performance. More specifically, the basic advantage of the proposed system, especially as far as the amperometric biosensor is concerned, is that the inhibition of the enzyme by the pesticide is reversible, thus ensuring the recovery of the biocatalytic membrane without the need for “reactivation” procedures. In addition to this, the proposed method is based on the activity of one enzyme only, with a reduced complexity of the system if compared to “traditional” biosensors for pesticides (primarily among them the acetylcholinesterase/choline oxidase sensors) and, at the same time, an improved activity of the enzymatic membrane, resulting in a shorter response time.

As can be seen from data reported in Tables 1, 3 and 4, the measurements performed in a standard solution of pesticide showed a lower detection limit in the range of 0.5–6 μg l⁻¹, which is sufficient to propose the use of the devices presented here for the preliminary screening of liquid samples. Subsequently, the exact composition and the chemical structure of all contaminants may be obtained by more specific and sophisticated confirmation methods, usually based on chromatographic–spectrometric techniques (primarily GC–MS and HPLC–MS).

Further research will be oriented to verify the usefulness of the method presented here for the screening of pesticides in real matrices and also, in view of a possible application of the electrochemical devices presented here, integrated in a flow-through cell, as detectors for HPLC.

References