Enzymatic Inhibition Constant of Acetylcholinesterase for the Electrochemical Detection and Sensing of Chlorpyrifos

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Abstract

Infiltration into soils of pesticides used during agricultural production has led to the contamination of aquatic ecosystems due to their long persistence in the environment. Some pesticides (e.g. Chlorpyrifos) are inhibitors of cholinesterase enzyme activity and their presence in water samples can be indirectly detected by a decrease in enzymatic activity. Biosensors based on cholinesterase enzymes are an alternative for the sensitive detection of important contaminants in the environmental sector. Acetylcholinesterase enzyme (AChE) catalyzes the hydrolysis of acetylthiocholine (ATCh) to produce thiocholine (TCh). This feature can be employed to measure the decrease in AChE activity. The inhibitory characteristics of the AChE-Chlorpyrifos system have been studied through cyclic voltammetry, by evaluation of the oxidation of the thiol group, which corresponds to TCh production on platinum electrodes in the presence of an inhibitor. In the present study, enzymatic curves were obtained at different concentrations of substrate and inhibitor, which were then used to determine the enzymatic kinetics corresponding to a mixed inhibition type, with an inhibition constant (Ki) of (18.26 ± 0.01) μM. TCh electrochemical detection appears to be a promising option for the development of biosensors to identify and quantify pesticides present in the ecosystem.
1. Introduction

The increasing use of pesticides in agricultural production, has led to the contamination of aquatic ecosystems due to their long persistence in soil environments and harmful impacts upon organisms [1], [2]. Organophosphorus and carbamate pesticides are highly toxic compounds. Chlorpyrifos [O, O-diethyl-O-(3, 5, 6-trichloro-2-pyridyl) phosphorothioate] is the most widely used organophosphate insecticide [3], its presence can be harmful to human health as it inhibits some important enzymes such as acetylcholinesterase (AChE), which is essential for the functioning of the central nervous system [4], [5]. AChE catalyzes hydrolysis of the neurotransmitter acetylcholine. As a result of AChE inactivity, acetylcholine is accumulated, causing the nerve order to be reiterated, leading to exhaustion, respiratory paralysis and death [5], [6].

The enzyme inhibition mechanism comprises the formation of a complex through a reversible/irreversible reaction of organophosphorus pesticides with AChE active sites [7]. An enzymatic inhibitor lowers the substrate binding capacity due to direct interaction between the enzyme and the inhibitor. In mixed inhibition, the inhibitor most often binds to a remote site and induces a conformational change that affects the active site, reducing the catalytic turnover and altering the substrate binding capacity [8], [9]. One approach to understanding the mechanism of action of enzymatic inhibitors has been to study the effect of inhibitor concentration on the enzyme reaction rate. The dissociation constant of the enzyme-inhibitor complex (Ki) has been used to describe the extent of inhibition [10].

Ki is proportional to the affinity and the inhibitory capability of the inhibitor from the enzyme. Therefore, it is a fundamental parameter when comparing the inhibition potency of pesticides and assessing the sensitivity of enzymes. The Ki value is different depending on the pesticide, the enzyme conditions and the type of entrapment in the case of immobilized enzymes [11].

Chromatographic techniques, such as gas chromatography (GC) and high-performance liquid chromatography (HPLC) offer high accuracy and sensitivity for compound monitoring; however, these techniques require highly trained personnel, are time consuming and limited to laboratory analysis [7], [12]. Thus, modern technologies have been developed to perform on-site analysis and obtain reliable and quick results for measuring pesticides.

In recent years, the use of AChE and acetylthiocholine (ATCh) in electrochemical biosensors has shown satisfactory results for pesticide analysis [13], [14], while the use of nanomaterials such as gold-platinum bimetallic nanoparticles [15], gold nanoparticles [16], and multiwalled Carbon nanotubes [17], coupled with AChE biosensors, has resulted in improved electrochemical performance. AChE inhibition-based biosensors are also portable, less expensive and they do not require complicated sample pretreatment [7], [12].

In order to study the electrochemical behavior of the AChE/ATCh system, cyclic voltammetry (CV) allows analysis of the responses from the oxidation of the thiol group of thiocholine (TCh), hydrolysis product of ATCh; where the current peak generated is proportional to the concentration of TCh and is reduced in the presence of an inhibitor, such as Chlorpyrifos [13], [18]. The aim of the present study is to determine the enzymatic inhibition constant for the AChE-Chlorpyrifos complex to use it in the electrochemical determination of these pesticides.

2. Materials and Methods

2.1. Chemicals and reagents

Acetylcholinesterase enzyme (AChE) extracted from electric eel, acetylthiocholine chloride (ATCh), Tris HCl and potassium chloride (KCl) were acquired from Sigma–Aldrich, (San Jose, Costa Rica). Deionized water was used in all experiments.

2.2. Electrochemical Cell

Electrochemical measurements were performed using an Autolab PGSTAT 302N (AUTOLAB, Netherlands) with a three-electrode system: Ag/AgCl (KCl, 3M) was used as the reference electrode and a Platinum sheet as the counter electrode, while Metrohm Screen Printed Platinum electrodes (AUTOLAB, Netherlands) were used as the working electrode. Cyclic voltammetry measurements were carried out by scanning from -0.2 to 1.0 V with a 0.2 V/s scan rate with a ±0.1 µA resolution.

2.3. Enzymatic Reactions

An electrochemical cell was prepared containing KCl (0.2M) and (10.80 ± 0.02) µl of AChE enzyme (139 U/ml), where one enzyme unit is defined as 1 mol of thiol group/min [19]. ATCh and Chlorpyrifos were added to the electrochemical cell according to a full factorial 3x3 design of experiments (DOE). Using Minitab 17 Software (Pennsylvania, USA) and based on preliminary experiments where a standard deviation was estimated, the minimum sample size was found to be of 2, this was obtained by using a two-sided analysis with a 95 % confidence interval with and error of 1. Measurements were recorded at all possible combinations, choosing two experimental factors: ATCh and Chlorpyrifos concentration in the electrochemical cell. To complete the DOE three different levels were chosen for each experimental factor.

Pesticides were added by determining the concentration required to inhibit approximately 50 %, 10 % and 0 % of the enzyme activity under the assay conditions. Table 1 shows the values for each factor at each level of the experiment design. Enzymatic reactions were followed in accordance with all possible combinations in the design of experiment for 10 minutes each, recording 13 measurements during that period.

Table 1: Design of a full factorial 3x3 design of experiments.

<table>
<thead>
<tr>
<th>Experimental Factor</th>
<th>Level 1</th>
<th>Level 2</th>
<th>Level 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCh Concentration</td>
<td>(275.00 ± 1.84) µM</td>
<td>(750.00 ± 3.25) µM</td>
<td>(1500.00 ± 6.26) µM</td>
</tr>
<tr>
<td>Chlorpyrifos Concentration</td>
<td>0.0 µM</td>
<td>(14.90 ± 0.05) µM</td>
<td>(44.80 ± 0.12) µM</td>
</tr>
</tbody>
</table>
3. Results and Discussion

Figure 1 shows the cyclic voltammograms corresponding to three different measurement times, in the system containing 1500 μM ATCh and no inhibitor. Curves b and c have an irreversible oxidation peak near 0.7 V which is associated with the oxidation process of thiocholine, a hydrolysis product of ATCh by AChE activity [18]. One important aspect when analyzing Figure 1 is the fact that the oxidation peak is not visible in curve a, this is because that curve was measured at the very beginning of the reaction time, when the enzymatic hydrolysis was barely starting and no appreciable thiocholine was produced. However, as the reaction time increased, the peak became visible and showed an increasing associated current, leading to the curve with the longest enzymatic reaction time, curve c, with the highest associated current. This behavior is related to the increasing thiocholine concentration as a result of AChE hydrolysis.

![Figure 1: Cyclic Voltammograms of measurements in (1500.00 ± 6.26) μM ATCh without inhibitor at a) 0 min of incubation, b) 1.5 min of incubation and c) 10 min of incubation.](image)

Figure 2: AChE activity behavior curve of current measurements at different ATCh concentrations: (●) (1500.00 ± 6.26) μM (▲) (750.00 ± 3.25) μM and (○) (375.00 ± 1.84) μM.

Figure 3: AChE activity behavior curves of current measurement with A) 14.9 μM and B) 44.8 μM of inhibitor at different substrate concentrations: (●) (1500.00 ± 6.26) μM (▲) (750.00 ± 3.25) μM and (○) (375.00 ± 1.84) μM.

The effect of the substrate and inhibitor concentration on the reaction kinetics catalyzed by free AChE was studied using ATCh chloride and Chlorpyrifos as substrate and inhibitor, respectively. Comparison of the measured current in an enzymatic system provides information about the interaction between the enzyme and substrate. These measurements may vary depending on the substrate concentration as shown in Figure 2, which shows the typical enzymatic behavior of the substrate concentration over time.

AChE activity measurements were used to evaluate the inhibition process resulting from the presence of Chlorpyrifos. To obtain the inhibition constant for the system, an incubation time of 10 min was selected for the measurements. Figure 3 shows enzymatic curves for 14.9 μM and 44.8 μM of inhibitor, respectively. In each figure, ATCh concentration is also varied.
The results show that by increasing the inhibitor concentration in the AChE-ATCh solution, the maximum current measured decreased, indicating that the enzyme has a lower affinity for the substrate, which is caused by the competition between the substrate and inhibitor for the active site, causing the loss of enzyme flexibility [6]. These responses are associated with AChE inhibition and the quantity of pesticide added (Figure 3). The decrease in current can be explained as a result of the blocking of the serine hydroxyl group on the enzyme structure by covalently bonding to the phosphate group, thus reducing the charge of the catalytic active site [19]. ATCh is reduced to TCh. The peak of anodic oxidation of TCh should increase according to the concentration of substrate added to the electrochemical measurements [17]. However, in the experiment with 14.9 μM of chlorpyrifos, no current change was observed compared to 750 μM and 1500 μM of ATCh (Figure 3A). Unfortunately, this result indicates that the acetylcholinesterase reaction did not occur normally.

Figure 2 and Figure 3A show that the concentration of 14.9 μM of chlorpyrifos does not have a significant inhibitory effect against AChE, and as a result, the measured current did not decrease significantly. In this study, the calculated Ki value is slightly higher than that of 14.9 μM. A lower concentration of pesticide comparing with Ki is hardly detectable. AChE immobilization is a strategy to build biosensors to detect pesticides in aqueous systems [20]-[22]. Therefore, the inhibition process may provide a method to quantify the amount of pesticides in real samples and Ki establishes an approximate value for the lower detection limit for AChE biosensors.

Figure 4 shows the Lineweaver-Burk plot, which is the double reciprocal plot of the enzyme reaction velocity (V) versus substrate concentration (1/V versus 1/[S]). There are four types of reversible inhibition: competitive, noncompetitive, uncompetitive and mixed inhibition. Each may be determined by graphical means using the Lineweaver-Burk plot [23]-[25]. For this study, it can be seen that the data line on the plot intersect in the second quadrant, under these conditions, exhibits a characteristic behavior of fully mixed inhibition. Therefore, the substrate must be competing with the analyte for the active enzymatic sites and for other sites in the enzyme structure. According to this, inhibition at low analyte concentrations could not be detected.

For pesticide analysis one of the most influential parameters is time [7]. Beyond 3 minutes of inhibition time, the curve tends toward a stable value, indicating the binding interaction between the pesticide and the enzyme, which reaches saturation (Figure 3). This change in current reflected the alteration of enzymatic activity, which resulted in a change of the interactions with the substrate. However, the enzyme was not totally inhibited, which was likely due to the binding equilibrium between pesticide and binding sites on the enzyme [12]. For AChE inhibition studies, the inhibition constant (Ki) provides a measurement of the dissociation (or binding) of the enzyme-inhibitor complex, and it is dependence on the structural and steric properties of the molecule. It is regarded as a useful parameter for the estimation of the inhibitory potency of a pesticide to AChE [26].

In our study, it was determined that the inhibition constant for the AChE-Chlorpyrifos complex was 18.26 ± 0.01 μM (Figure 5). Ki may be regarded as an affinity constant, and therefore, the smaller the value of Ki, the tighter the complex. Pesticides with small values for Ki are strong inhibitors, and those with large Ki values are weak inhibitors [27]. In this regard, when comparing this to other previously reported inhibition constants for AChE (Table 2) [28], Chlorpyrifos acts as a strong inhibitor. Previous studies explain that Chlorpyrifos has an adequate fit to the enzyme’s active sites, making it a potent pesticide [29], [30].

### Table 2: Ki values for inhibition of AChE from different organophosphate pesticides.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ki value (μM)</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Diazoxon</td>
<td>0.79</td>
<td>[28]</td>
</tr>
<tr>
<td>Malaoxon</td>
<td>5.6</td>
<td>[28]</td>
</tr>
<tr>
<td>Isomalathion</td>
<td>7.2</td>
<td>[28]</td>
</tr>
<tr>
<td>Malathion</td>
<td>130</td>
<td>[28]</td>
</tr>
</tbody>
</table>

Figure 5: Inhibition constant (Ki) according to inhibitor concentration and reaction velocity: (■) (1500.00 ± 6.26) μM (●) (375.00 ± 1.84) μM.
4. Conclusions

In this study, the inhibition between Chlorpyrifos and Acetylcholinesterase is found to be a fully mixed inhibition type, with a characteristic inhibition constant with a value of $(18.26 \pm 0.01)$ μM. These results represent a useful tool for the study of the extent of inhibition and the mechanism of action of enzymatic inhibitors. It also sets an approximate value for the lower detection limit of biosensors based on AChE immobilization, as their application is important to enhance the sensitivity of enzymatic biosensors in pesticide detection.

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References


