Talanta 111 (2013) 1–7

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Effect of chlorpyrifos on the inhibition of the enzyme acetylcholinesterase by cross-linking in water-supply samples and milk from dairy cattle

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ARTICLE INFO

Article history:
Received 9 November 2012
Received in revised form
13 March 2013
Accepted 15 March 2013
Available online 23 March 2013

Keywords:
Acetylcholinesterase
Biosensor
Enzyme
Pesticide
Screen-printed electrodes

ABSTRACT

A methodology for the determination of chlorpyrifos in water-supply samples and in milk from dairy cattle was developed. An amperometric biosensor was used to inhibit the enzyme acetylcholinesterase (AChE), which was immobilized by the cross-linking method (crosslinks between the enzyme and the sensor). The potential applied, the amount of enzyme to be immobilized and the acetylthiocholine (ACTh) concentration were optimized before calibration and analysis of the samples was performed. The concentration of chlorpyrifos was determined in the range of 1.0×10^{-6} M to 5.0×10^{-2} M with a detection limit of 5.0×10^{-6} M. Spiked water samples showed high recoveries (91.32% and 93.98% for low and high chlorpyrifos levels, respectively), while milk samples exhibited a matrix effect with recoveries of 82.81% and 79.77% for high and low chlorpyrifos levels, respectively. The average concentration of chlorpyrifos in the water supply samples (5.11×10^{-6} M), determined using the biosensor, was compared using gas chromatography and gave an average value of 3.04×10^{-6} M. The results allow it to be concluded that although chromatographic methods are still more exact, biosensors are promising tools for the determination of analytes in the field, as they have a low cost, a reduced analysis time and good reproducibility in the data.

1. Introduction

Pesticides are widely used chemicals, especially in agriculture. Their extensive use has led to the contamination of water sources, and they have had a major impact on health and the environment [1]. This is especially true for organophosphorus pesticides which are used for pest control and other domestic purposes [2]. Chlorpyrifos is still one of the most commonly used organophosphorus pesticides. Due to agriculture and point source discharges, this pesticide is principally responsible for the toxicity of a large part of aquatic life [3]. However, in recent years it has been shown that chlorpyrifos may not only be present in water bodies, but also but also in livestock sites, as cattle herds could drink water from contaminated waterways. This leads to the production of milk contaminated with the aforementioned pesticide. Therefore, the quality of milk from these cattle herds must be monitored. Biosensors can be an effective and simple tool for monitoring contaminants such as chlorpyrifos in milk [4] and water.

Biosensors are analytical devices that use the sensitivity and selectivity of a bioreceptor adhered on the surface of a transducer. The transducer is able to respond and transform a biochemical and/or physicochemical property into a measurable signal as a result of recognition between the bioreceptor and target analyte [5]. These are coupled to elements such as biological sensing enzymes, antibodies, microorganisms or DNA, and integrated into transducers that can be electrochemical and optical, among other types [6]. Amperometric biosensors are based on measuring changes in the current of the working electrode due to oxidation or reduction of metabolic products or intermediates generated in biochemical reactions [7,8].

Chlorpyrifos is considered a neurotoxic compound that irreversibly inhibits the enzyme acetylcholinesterase (AChE), essential for the functioning of the central nervous system in humans and insects. This results in the accumulation of the neurotransmitter acetylthiocholine (ATCh), which interferes with muscular responses and vital organs, causing severe symptoms and eventually death [9]. When AChE is immobilized on the surface of the working electrode (SPEs), its interaction with the substrate produces an electroactive species. In this process, the acetylthiocholine (ACTh) can replace the original substrate of AChE, and therefore, the ACTh is hydrolyzed in the same way as the original substrate, producing thiocholine (TCh) and the corresponding carboxylic acid (acetic acid in this case) [5]:

$$\text{acetylcholine} + H_2O \xrightarrow{\text{AChE}} \text{thiocholine} + \text{acetic acid}$$

$$2\ \text{thiocholine} \xrightarrow{\text{oxidation}} \text{dithio–bis–choline} + 2H^+ + 2e^-$$

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http://dx.doi.org/10.1016/j.talanta.2013.03.036

In this study, an enzymatic amperometric biosensor was used in order to establish a methodology for the rapid detection of the pesticide chlorpyrifos in water supply samples and milk from dairy herds. The methodology was based on the inhibition of acetylcholinesterase enzyme activity (AChE), which was immobilized using the cross-linking method on the surface of Screen Printed Electrodes (SPEs).

2. Materials and methods

2.1. Reagents

Acetylcholinesterase from Electric eel (AChE, specific activity of 500 U/mg solid), acetylthiocholine chloride (ATCh) with a purity ≥99%, Albumin from bovine serum (BSA, Cohn V Fraction > 96%), glutaraldehyde (GA, grade I, 8%), Nafion® (Perfluorinated membrane 5 wt%), 5,5′-dithio-(2-nitrobenzoic acid) (DTNB) and sodium monohydrogen phosphate (PBS, Na_2HPO_4) were purchased from Sigma–Aldrich Co. (Stein-Heim, Germany). The AChE, ATCh, GA and DTNB were kept at −20 °C. All solutions were prepared with ultrapure water (average conductivity 0.04 μS/cm). Chlorpyrifos standard solution was purchased from Dr. Ehrenstorfer, GmbH (Germany) with a purity ≥99%. Stock solutions were prepared by dissolution of the Chlorpyrifos standard solution in HPLC grade methanol (Merck) and kept at −20 °C for a maximum of 1 week.

2.2. Amperometric biosensor

Amperometric measurements were carried out with a SPE AmpBio device PS, (Biosensor Srl, Formello, Rome, Italy), with dimensions of 22.0 cm wide × 15.0 cm long × 10.0 cm high, and composing of two measuring cells in parallel designed for the measurement of Screen Printed Electrodes (SPEs). The biosensor operated under a flow of 200 μL/min, with a current of 5 μA and a variable voltage of +100 to +400 mV, in agreement with the experimental design, and connected online to a computer (Hewlett Packard) for the acquisition of information (Fig. 1(a). BIOCOM software was used (Biosensor S.r.l, Formello, Rome, Italy).

Screen Printed Electrodes (SPEs) were purchased through the company DropSens, SL (Spain), with dimensions of 3.4 cm long × 1.0 cm wide and 0.05 cm thick. Contacts of the reference electrode and the connection electrodes were silver and both the working electrode and the auxiliary electrode (with a diameter of 4 mm) were made of carbon (Fig. 1(b).

2.3. Free enzyme activity

Determining the activity of the free enzyme AChE was carried out in a Thermo (EVO EVON 600LC-237 001) spectrophotometer, in accordance with the method described by Ellman et al. [10] and modified by Nunes et al. [11], which is based on two coupled reactions [12]:

$$\text{acetylthiocholine (ATCh)} \xrightarrow{\text{Acetylcholinesterase(AChE)}} \text{acetic acid} + \text{thiocholine}$$

$$\text{thiocholine} + 5,5'\text{dithio-bis-2-nitrobenzoic acid(DTNB)} \rightarrow \text{2-nitrobenzoate-5-mercaptothiocholine} + \text{5-thio-2-nitrobenzoate}(TNB^{2-})$$

The final TNB^{2-} product is yellow coloured and can be detected optically at 412 nm.

In accordance with the procedure established by Nunes et al. [11], the following volumes were added sequentially in a quartz cuvette: 200 μL of DTNB (2.5 mmol/L), 350 μL of PBS (2 mmol/L, pH 8.0), 50 μL of the enzyme/pesticide solution previously incubated at 30 °C, and 200 μL of ACTh (2 mmol/L). The volumes were determined spectrophotometrically at 412 nm. Aliquots of the enzyme/pesticide solution were taken at intervals of 2 min over a 20 min period. Assays in the absence of both the pesticide and the enzyme/pesticide solution were used as controls and blanks, respectively. Finally, the biomolecular constant (k_i) was determined.

2.4. Protocol for amperometric analysis

2.4.1. Immobilisation of the enzyme in SPEs

The AChE was immobilized using the cross-linking method, which involves the use of bifunctional reagents such as glutaraldehyde that cause intermolecular links between the enzyme and the transducer [13–15]. To carry out the immobilization, 5 μL of GA (0.25% *w/v*) were placed on the working electrode (SPEs) and allowed to dry at 4 °C, then 3 μL of a solution containing 30 μL of BSA (5% w/v), 30 μL of Nafion (1% w/v) and 30 μL of AChE (units of enzyme used varied depending on the design of the experiments) were added and allowed to dry again at 4 °C. The SPEs were stored in 2 mmol/L PBS at pH 8.0 and 4 °C for at least 3 days before the testing began [9]. This was because according to studies by Dou et al. [16] and Hildebrandt et al. [2], AChE has an optimum working pH in the range of 7.0 to 8.5 with a maximum response at pH 8.0. The SPEs were stored under conditions of hydration since when stored dry, the enzyme is rapidly denatured and loses nearly all its activity, giving very low, almost undetectable, signals. Prior to analysis, the SPEs were regenerated for 10 min with PBS (2 mmol/L, pH 8.0) with stirring [9] at room temperature (22 °C).

2.4.2. Optimization assays

In order to find the best conditions for validation, several experiments were developed in which the voltage, units of the AChE enzyme, and mmol/L of ACTh were varied in different proportions. The first test was performed keeping the ATCh and AChE constant (0.12 U and 3 mmol/L, respectively) and varying the voltage applied at rates of +100 mV, +200 mV, +300 mV and +400 mV. In the second assay the AChE was varied in proportions of 0.06 U, 0.12 U and 0.24 U, but the ACTh remained constant at 3 mmol/L and the voltage used was that which gave the best response in test 1. Finally, in a third experiment the ATCh was varied in proportions of 1 mmol/L, 3 mmol/L, 5 mmol/L, 7 mmol/L and 9 mmol/L but the AChE and voltage remained constant in accordance with the best results from experiments 1 and 2. Assays were performed in triplicate and new biosensors were employed in each case to avoid problems with reproducibility and data response.

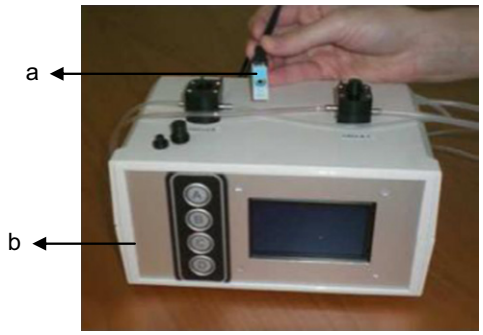

Fig. 1. Amperometric biosensor (SPE AmpBio PS) used for the determination of chlorpyrifos. (a) Amperometric unit. (b) Screen Printed Electrodes (SPEs).