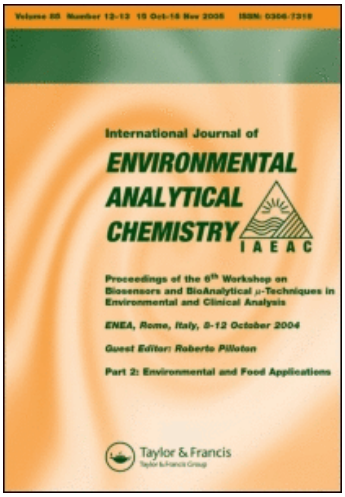

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Lipase biosensor for tributyrin and pesticide detection

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Lipase biosensor for tributyrin and pesticide detection

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Potentiometric biosensors based on *Candida rugosa* lipase was described for the detection of organophosphorus pesticide; methyl-parathion and tributyrin. Lipase was immobilized on the glass electrode by means of a gelatin membrane, which is then cross-linked with glutaraldehyde. The principle of the biosensor is based on the measurement of pH variation which was recorded in millivolts due to the enzymatic hydrolysis of tributyrin to butyric acid. For the inhibitor detection, biosensor responses were measured after pesticide treatment, which caused a drop in enzyme activity because of the irreversible inhibition. Reactivation conditions of the reused enzyme electrodes were also investigated by pyridine-2-aldoxime methiodide (2-PAM). The limit of detection for tributyrin was estimated as 93 μM for lipase sensor within the linear range of 65–455 μM.

Keywords: *Candida rugosa* lipase; Biosensor; Organophosphorus pesticide; Tributyrin

1. Introduction

Lipases or triacylglycerol acyl ester hydrolases are enzymes with an intrinsic capacity to catalyse the cleavage of carboxyl ester bonds in tri-, di-, and monoacylglycerols (the major constituents of animal, plant, and microbial fats and oils). As a result of this type of reaction, carboxylic acids and alcohols with a lower number of ester bonds (and eventually glycerol) are released [1]. These enzymes are very important because of their broad specificity towards different subsrates. Nowadays, lipases are utilized in many different applications, e.g. in the regioselective modification of polyhydroxylic compounds, modified oils and fats, food additives and flavours making pharmaceutical products, biodetergents, cosmetics, perfumes, new biopolymeric materials, biodiesel, agrochemicals, biosensors, pesticides, etc. [2–9].

Organophosphorus (OP) compounds are among the most toxic substances and are thus commonly used as pesticides, insecticides, and chemical warfare agents. Early detection of OP neurotoxins is important for protecting water resources and food supplies, as a defence against terrorist activity and for monitoring detoxification processes [10].

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Until recently, the identification and quantification of pesticide residues in water and other sources have been limited to the traditional chromatographic and spectroscopic methods. Although they are very sensitive, these sophisticated techniques are time-consuming and require highly trained personnel and expensive apparatus. Furthermore, these methods are unsuitable for real-time, *in situ*, or on-line monitoring of pollutants. Considerable attention is now being given to the development of biosensors that can provide continuous, *in situ*, and rapid measurement of various compounds. A biosensor can be defined as a device resulting from the association between a sensitive biological element and a transducer, which converts the biological signal into a measurable physical signal [11]. Biosensors are especially useful for inexpensive on-line monitoring [12, 13].

Triglyceride detection and estimation is a clinically important parameter that is correlated to heart disease [14]. For the same reason and increased health awareness among people, it is important to estimate the triglyceride content in foods. There is an immense scope for improving the sensitivity and the ease of operation of commercial sensors presently used for estimating triglycerides [15].

In this work, a potentiometric biosensor based on *C. rugosa* lipase was developed and applied for the detection of both, methyl-parathion and tributyrin.

2. Experimental

2.1 *Materials*

Lipase from *C. rugosa* (EC 3.1.1.3), calf skin gelatin, and pyridine-2-aldoxime methiodide (2-PAM) were purchased from Sigma Chemical Co. All other chemicals were analytical reagent grade.

2.2 *Biosensor preparation*

Lipase (1 mg, 887 unit mg^{-1} for olive oil) and gelatin (20 mg) were dissolved in 250 μL of phosphate buffer (pH 7.5, 50 mM). The mixture was incubated at 38°C for 5 min, then dropped on the surface of the glass electrode. The electrode was allowed to stand at 4°C for 45 min then cross-linked with 2.5% glutaraldehyde solution [in phosphate buffer (pH 7.5, 50 mM)] for 5 min.

2.3 *Measurement*

The biosensor response was measured by using a 718 Titrino pH stat following the principle of pH changes in the bioactive membrane due to the formation of butyric acid as a result of enzymatic tributyrin hydrolysis as shown below and recorded in millivolts. A standard solution of tributyrin (65 mM) was prepared in 100 mM NaCl. Tween 40 was used for emulsification of tributyrin.

Different amounts of substrate were added to 25 mL of 100 mM NaCl at 30°C, and the responses were monitored.

$$\underset{\text{(tributyrin)}}{C_{15}H_{26}O_6} + \underset{\text{(water)}}{3H_2O} \rightleftharpoons \underset{\text{(glycerol)}}{C_3H_8O_3} + \underset{\text{(butyric acid)}}{3C_4H_8O_2}.$$

The inhibitory effect of the organophosphate pesticide methyl-parathion on the lipase biosensor was evaluated by determining the decrease in millivolts observed for the hydrolysis of the substrate as given above. In order to perform this, the biosensor was first incubated in the pesticide solution for a certain period (incubation time) and then rinsed with distilled water. After that, the response toward the substrate was measured as described above, and the degree of inhibition was calculated as a relative decay of the biosensor response (equation (1))

$$I(\%) = \left[\frac{(E_o - E_i)}{E_o}\right] \times 100\%, \tag{1}$$

with E_o and E_i as the response values prior to and after incubation, respectively. Plots of percentage inhibition values corresponding to –log [methyl-parathion] were drawn for each incubation period.

2.4 *Stability*

Investigation of operational stabilities is one of the most critical parameters in biosensor studies. As far as inhibitor detection is concerned, it is very important to know the reason for the decrease in the activity due to the presence of inhibitor apart from the external effects.

Biosensor responses can decrease as the biosensor is repeatedly used. Operational stability tests were carried out with a 390×10^{-3} mM standard tributyrin solution at 30°C by making subsequent measurements over 430 min.

2.5 *Reactivation of electrodes and reusability*

In the case of organophosphate pesticides which are irreversible inhibitors, one of the main requirements is to reactivate the inhibited enzyme in order to allow for continuous monitoring. A few reports concerning the re-activation of inhibited enzyme electrodes have been published [16–18]. The reactivation of the enzyme is performed by using a powerful nucleophilic reagent such as 2-PAM.

3. Results and discussion

Microbial lipase constitutes an important group of biotechnologically valuable enzymes, mainly because of the versatility of their applied properties and ease of

mass production. Microbial lipases are widely diversified in their enzymatic properties and substrate specificity, which make them very attractive for industrial applications [19]. A biosensor based on the enzyme-catalysed dissolution of biodegradable polymer films has been previously developed. Potential fields of application of such a sensor system were reported as the detection of enzyme concentrations and the construction of disposable enzyme-based immunosensors, which employ the polymer-degrading enzyme as an enzyme label [20]. Furthermore, it is reported that radiolabelled polynucleotide probes have been employed extensively for the detection of complementary nucleic acids by specific hybridization. Within the last few years, various methods have been developed using enzyme-labelled probes to avoid unstable and hazardous isotopes. By screening various hydrolytic enzymes to fit the special demands, fungal lipases turned out to be the most practical [21].

Lipases could be immobilized onto pH/oxygen electrodes in combination with glucose oxidase, and these function as lipid biosensors [22] and may be used in triglycerides [23] and blood cholesterol determinations [24]. This work presents the first application of a *C. rugosa* lipase-based biosensor for both inhibitor and triglyceride detection.

3.1 *Optimization of enzyme quantity*

Electrodes were prepared using different amounts of *C. rugosa* lipase. Responses for different tributyrin concentrations were measured for each electrode, and the calibration curve for the electrodes (which include the optimum enzyme quantity) is shown in figure 1. Since higher responses were obtained by the enzyme electrode containing 1 mg of enzyme, this biosensor was chosen as the optimum and used for further experiments.

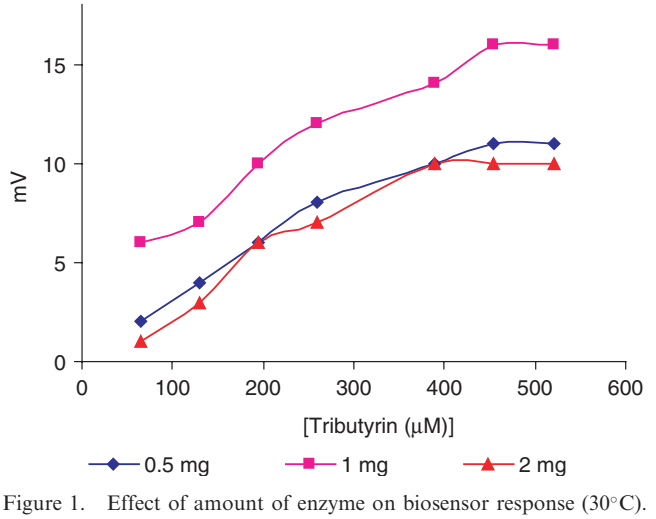

Figure 1. Effect of amount of enzyme on biosensor response (30°C).

3.2 *Analytical characteristics*

The effect of the substrate concentration on the electrode response was investigated. It is well known that selection of a proper substrate concentration is important in enzyme inhibition studies. The inhibition level was evaluated from the response of the active and inhibited forms of the enzyme. Hence, all the enzymes have to take part in the reaction that corresponds to the saturation part of the calibration graph. The saturation of the enzymatic membrane with the substrates contributes to the most sensitive detection of the inhibiting effect [25]. For this reason, the saturation concentrations of tributyrin were chosen for the biosensor design. The calibration graph is shown in figure 2. The plot of mV values against corresponding substrate concentrations (μM) showed a linear fit ($y = 0.0253x + 4.4286$) with a slope of 0.0253 (R^2; 0.97) for the detection range (65–455 μM) with standard solution of tributyrin. The LOD value was determined as 93 μM.

As far as repeatability was concerned, the standard deviation (SD) and coefficient of variation (CV) were calculated by repetitive measurements of biosensor response with tributyrin (390 μM), and these values were found as (±14.94 μM) and 4.5%, respectively (n; 7).

Operational stability was also investigated as already mentioned in the experimental section. The results showed that the biosensors worked without any decrease in activity after 20 measurements with tributyrin over 430 min.

3.3 *Pesticide detection*

In order to monitor the inhibition effect of pesticide concentration on sensor response the enzyme electrodes were incubated with different methyl-parathion concentrations for different periods (15–60 min). Calibration graphs for each time period were drawn,

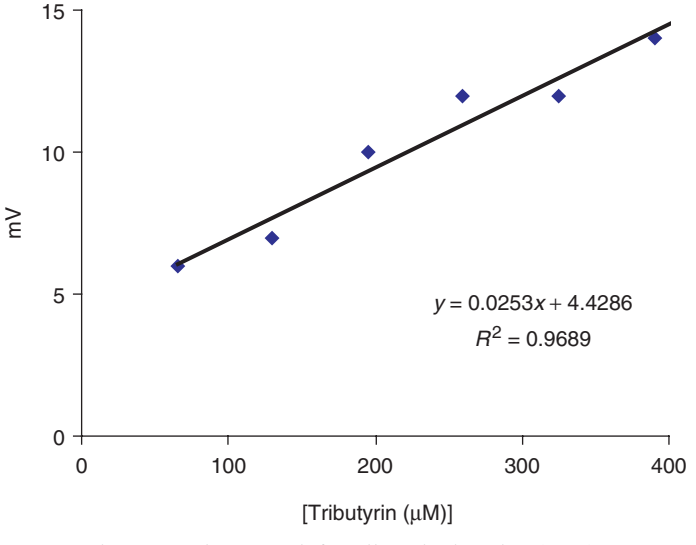

Figure 2. Linear graph for tributyrin detection (30°C).

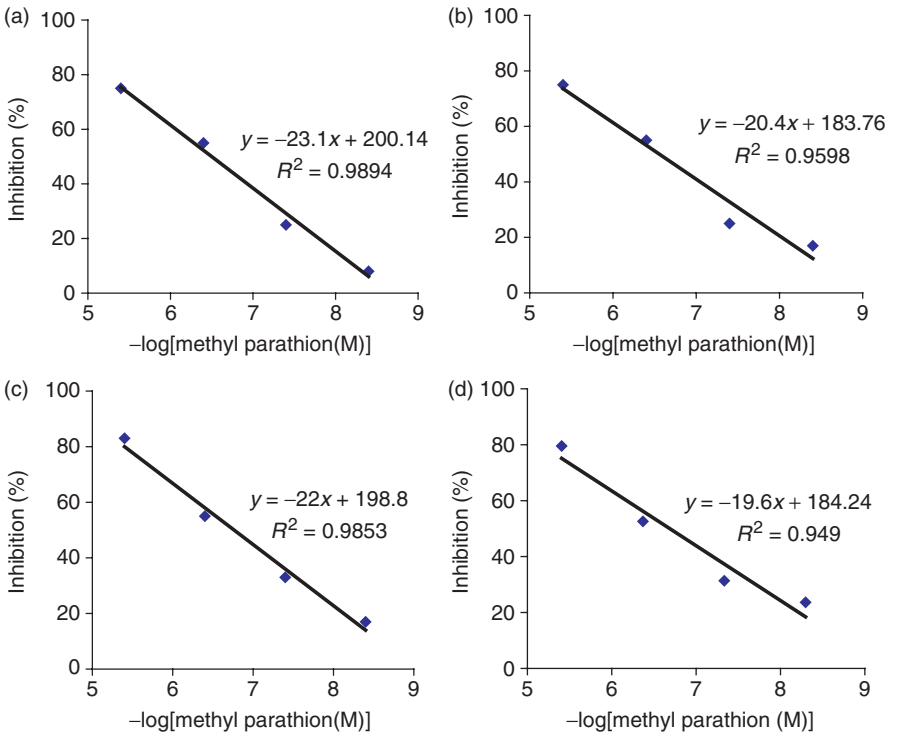

Figure 3. Inhibition curves *vs.* methyl-parathion concentrations at different incubation times: (a) 15 min, (b) 30 min, (c) 45 min, and (d) 60 min (at 30°C).

and these are shown in figure 3. According to the data, 15 min of incubation time is enough to obtain a proper linearity, and no significant changes were observed in activity for further incubation times.

The enzyme electrodes were reactivated with PAM solution (1 mM) for different periods after the inhibition with methyl-parathion (data not shown). Responses after treatment with pesticide and PAM were measured, and relative activities were calculated. Since full regeneration was not observed with 1 mM of PAM solution, the inhibited electrodes were treated with more concentrated PAM (5 mM) to provide higher reactivation levels. Figure 4 shows the reactivation curve of pesticide inhibited electrodes by PAM solutions at 1 and 5 mM. After treatment of 4 μM methyl-parathion, the enzyme electrode was incubated with reactivation solution, and can be clearly seen that the recovery of enzyme activity was observed within 30 min by 5 mM of PAM; when using 1 mM of PAM, only 70% of activity could be recovered.

The fully reactivated electrode was used subsequently for pesticide detection to investigate repeatability as an inhibitor biosensor. Data showed that the enzyme electrode could be used five times for pesticide detection by reactivating the electrode after each measurement. The SD and CV values were calculated as ±35.35 μM and 0.08%, respectively.

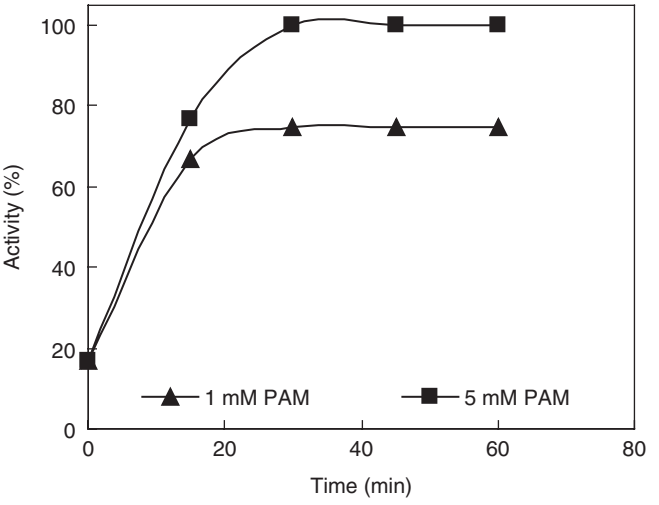

Figure 4. Reactivation curves of inhibited electrodes with PAM (enzyme electrode inhibited with 4 μM of methyl-parathion).

4. Conclusion

We have described the preparation of a potentiometric-based enzyme electrode to determine the concentrations of tributyrin and organophosphate pesticide. The detection process, using the immobilized biocomponent, was simple and allowed direct measurement of tributyrin. As far as inhibitor detection is concerned, it is possible to reactivate the inhibited enzyme by PAM treatment. The reactivation method proposed by the present research would provide more reliable pesticide detection with a reusable enzyme electrode. Generally, enzyme electrodes constructed for pesticide analysis have been based on cholinesterase enzymes using different modes of immobilization [26]. For biosensors for organophosphate compounds, the acetylcholinesterase (AChE) inhibition methods, using AChE modified amperometric [27], potentiometric [28], conductometric [29], and fibre optic [30, 31] transducers, have been reported. In this work, an alternative biosensor system was developed with lipase, which, similar to acetylcholinesterase, is a serine hydrolase, and this biosensor can be used for triglyceride detection.

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