Optimised Biosensors Based on Purified Enzymes and Engineered Yeasts: Detection of Inhibitors of Cholinesterases on Grapes

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ABSTRACT

Purified acetyl cholinesterase (AChE) from electric eel or engineered yeasts (Kluyveromyces lactis) expressing cholinesterase activity from rat, were immobilised on a nylon membrane for the measurement of residual cholinesterase activity after inhibition by the organophosphorous insecticide Paraaxon (diethyl p-nitrophenyl phosphate). Measurement was separately operated after the incubation (inhibition) step. The measurement of the residual enzyme activity was therefore performed in a standardised solution with a choline electrochemical biosensor, without any interference, avoiding the use of protecting selective membranes. Both commercial amperometric sensor and screen-printed electrodes, produced and optimised in the laboratory, were used. The time consuming incubation step was simultaneously performed on several samples, thus lowering the analysis time per sample. Good limit of detection (LOD)

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(0.1 μg/L) and reproducibility were obtained for the analysis of para-oxon. A simple procedure for the detection of cholinesterase inhibitors on grapes was then developed. The procedure itself could be further extended to several fruits and vegetables, giving a simple but effective tool to verify the absence of residues of anticholinesterasic insecticides.

**Key Words:** Cholinesterase inhibitors; Acetyl cholinesterase; Gas chromatography; Limit of detection; Enzyme inhibition-based biosensors.

### 1. INTRODUCTION

In the early 80s, organochlorine insecticides were progressively and significantly replaced by OP insecticides and derivatives of carboxylic acid insecticides, showing a relatively rapid degradation in the environment, but representing a serious risk because of their acute toxicity.\(^1\)\(^,\)\(^2\) Even if OP insecticides, in their turn, are being replaced by synthetic pyrethroids and other insecticides,\(^3\) characterised by a lower acute toxicity, their use is still largely diffused in agriculture because of their high effectiveness. As a consequence, adverse acute effects on living organisms are possible, caused by the inhibition of active sites of fundamental enzymes (i.e., cholinesterases involved in muscle physiology and in nervous system) and the consequent death of the most sensitive and vulnerable organisms, altering the equilibrium of aquatic ecosystems and the food chain.\(^4\) Main risks for people regard both the professional (workers in pesticide manufacturing industries and farmers due to pesticide agricultural usage\(^5\)\(^,\)\(^6\)) and not professional exposures such as domestic use,\(^7\)\(^,\)\(^8\) food,\(^9\) and fresh water contamination.\(^10\)

The European Drinking Water Act (1980) and the following recent national legislation, as a general approach, do not allow the concentration of these compounds in drinking water to exceed individually the limit of 0.1 μg/L, and severe limits are also imposed to the concentration of possible residues of these insecticides in fruit, vegetables and other kinds of food. Therefore, monitoring and control analyses at such a low concentration level, with both high sensitivity and selectivity, remain a topical issue. HPLC and GC (especially if coupled with MS detection) were showed to be very effective and highly sensitive methods\(^11\)\(^–\)\(^13\) for the analysis of these compounds, but require quite expensive instrumentation and skilled laboratories. ELISA kits are quite expensive too: they do not require very expensive instrumentation and can be used in field analysis and show high sensitivity,\(^14\) but are normally directed toward a specific compound (with minor residual problems of cross-reactivity). These concerns have stimulated research
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towards development of biosensing technology as a new tool for detecting herbicides\cite{15} and insecticides\cite{16,17} in a simple and cost effective way.

1.1. Research Background

Many AChE (and/or BChE) based analytical procedures and biosensors\cite{18–23} were already designed to detect OP insecticides (and other cholinesterase inhibitors). Several protocols for the measurement of AChE activity (reaction 1), using biosensors, have been set up. These protocols generally allow relatively fast, simple and cheap “screening” analyses by using differential pH metres, ISFET, LAPS, conductimetric cells, amperometric carbon modified electrodes or, as in this article, SPEs based on choline oxidase (ChOx) \cite{reaction (2)}. Reaction (1) is, in fact, catalysed by the enzyme AChE, that, in presence of OP insecticides is inhibited: the consequence of the inhibition is the yield reduction in reaction (2) (less H$_2$O$_2$ produced and measured by the sensor) directly correlated to the inhibitor (organophosphorous insecticide) in the sample.

\[
\begin{align*}
\text{Acetylcholine} + \text{H}_2\text{O} & \rightarrow (\text{AChE}) \rightarrow \text{Choline} + \text{Acetate} \quad (1) \\
\text{Choline} + 2\text{O}_2 + \text{H}_2\text{O} & \rightarrow (\text{ChOx}) \rightarrow \text{Betaine} + 2\text{H}_2\text{O}_2 \quad (2)
\end{align*}
\]

It is necessary to point out the limitations and possible advantages derived from this approach.

1. The measured “effect” (inhibition of AchE) can be caused by the presence in the sample of one or more “unknown” inhibitors. Consequently, it is not possible to identify the inhibitor/s and determine the concentration of any single specific pesticide in the sample using this kind of enzyme inhibition based biosensors.

2. The legislation limits for these contaminants are generally expressed as maximum allowed concentration of any single specific pesticide and consequently, considering point 1, results obtainable by the use of the enzyme inhibition based biosensors cannot be considered satisfactory for control analyses.

3. The measurement could be usefully expressed in a unit related to the toxicity effects. It was already proposed to use the “anticholinesterase inhibition” as a parameter of (water) pollution or, contrarily, as a “quality index”\cite{24–26}. This interesting index of toxicity could be measured with enzyme inhibition based biosensors and methods. Actually, this approach seems to take into account only the enzyme activity, overlooking many other
important parameters involved in the adsorption dynamic of OP in animals and humans as well as solubility, hydrophobicity, catabolic pathways of these compounds.\textsuperscript{[27,28]} In spite of all that, a quite surprising good linear correlation ($r^2 = 0.995$) was already found between ln(LD\textsubscript{50}) and the inhibition level of pure AChE for several OP.\textsuperscript{[24]}

For these reasons, this kind of biosensors could only be used as an EWS, useful for a preliminary analytical screening of many samples in a fast, cheap and simple way: absence of false negatives is therefore the only essential requirement.

Several aspects were investigated in the scientific literature.

The amount of initial AChE units greatly affects the LOD of this method: a low amount of the enzyme and a long incubation time will result in lower LOD. Several analysis formats have been proposed for the detection of AChE inhibition.

1. \textit{Biosensors with co-immobilised ChOx and AChE}. These biosensors are not very sensitive (LOD = 20 $\mu$g/L) because the measurement of AChE activity has to be performed without an incubation (inhibition) step. Moreover, the amount of AChE is not easily controlled on the tip of the sensor and the membranes with the immobilised enzymes have to be replaced after few analyses because of the irreversible inhibition of AChE. So, this kind of biosensors could be usefully used only as disposable analytical tools and for the analysis of samples, where high pesticide concentration is expected.

2. \textit{Biosensors with immobilised ChOx and free AChE in solution}. The amount of the AChE enzyme was minimised in a reproducible and easily controllable way, but serious problems took place due to adsorbed AChE on the tip of the electrode.\textsuperscript{[16,17]} After few analyses, AChE adsorbed onto the choline biosensor tip interfered with the measurement of the activity of the enzyme free in solution.

In both cases kinetic measurements were performed directly in the sample, with possible electrochemical interferences, which were minimised with selective membranes, this resulting in high practical LOD (typically 10 $\mu$g/L).\textsuperscript{[16,17]}

These problems were practically solved with the following analysis format.

3. AChE immobilisation on a separate membrane (placed in solution) provided a more convenient protocol of analysis separating the “inhibition” step from the residual activity measurement of AChE. The measurement was, therefore, performed in a clean standard solution allowing the biosensor
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to be used without any protecting membrane and exploiting, for this reason, better sensitivity. As previously reported,\(^1\) with a very low AChE amount (10–50 mU) immobilised on a nylon membrane, a very low LOD (0.1 \(\mu\)g/L for paraoxon) can be obtained, with high reproducibility. The possibility to use the same ChOx biosensor for several hundreds of analyses and a relatively short time of analysis make this approach probably the best one.

This approach, expressed in an optimised protocol of analysis, was applied in this article for measuring the residual activity of AChE immobilised onto nylon membranes with a ChOx modified SPE, in order to determine AChE inhibitors directly on grapes by using either a commercially available enzyme or a recombinant yeast,\(^{29,30}\) with a genetically expressed AChE activity.

Engineered biomolecules, in fact, represent a powerful approach to obtain simple artificial structures with new or improved properties (i.e., specificity, stability, sensitivity), useful for biosensors development.\(^{31}\) Several engineered cells are also suitable for biosensing purposes allowing low cost continuous production of enzymes on the tip of a sensor.\(^{32}\)

2. EXPERIMENTAL

2.1. Material, Chemicals, and Reagents

A commercial type amperometric sensor was used (IDRONAUT-Brugherio, Milan), with an Ag/AgCl (3 cm\(^2\) surface area) as RE, and a Pt wire (0.1–1 mm) as WE.

Screen-printed electrodes (SPEs)\(^{33}\) were obtained using a Fleischle (Brackenheim, Germany) screen printer on a PVC slide\(^{15}\) acceding to several layouts. Carbon inks for SPEs were improved by doping procedure: Pt (Aldrich 23.755-8) or Rh (Aldrich 20.616-4) absorbed on graphite powders were mixed with the carbon pastes (obtained from Acheson).

An homemade flow cell, used for setting up choline measurements, consisted of two blocks, where the SPE has been inserted and fixed by an o-ring, and a three-way inlet flow system for carrier and sample solutions.

Choline oxidase (oxygen 1-oxidoreductase, EC 1.1.3.17, extracted from Alcaligenes species, 10 units/mg solid, lyophilised form) was purchased from Sigma. AChE (EC 3.1.1.7, extracted from electric eel; 1000–2000 units/mg protein, lyophilised form) as well as crystalline form of AChCl, choline chloride and paraoxon were from Sigma. GA 25\% aqueous solution was obtained from Fluka. PAP polymer from Hercules Polycup. Selective hydrogen peroxide membranes (MWCO < 100 Da) were prepared in laboratory,\(^{15}\) nylon membranes were obtained from Pall Inc., Italia (Milan). Syringe for filtration
and 0.2-μm filters were purchased from Whatman Inc. Supporting electrolyte 0.1 M PB solutions were prepared from deionised water (ELGA-STAT deioniser). All other chemicals were of analytical grade and used without further purification.

2.2. Immobilisation of Pure Enzymes and Yeasts

2.2.1. Purified AChE Immobilisation on Nylon Membranes

AChE was immobilised on nylon-preactivated membranes by using a 12.5% GA solution in PB at pH 7.0, as crosslinking agent. The treatment consisted simply in immersing a piece of the membrane in GA solution for 1 hr, washing it in PB solution and dropping 3 μL of the enzyme solution (25 U/mL) with a fine μ-pipette tip normally used for sample application in gel electrophoresis and ensuring a large excess of the enzyme with respect to the number of aldehyde groups on the membrane surface. Adsorbed AChE, not covalently linked to the membranes, was removed by cool sonication (1 hr in PBS).

Twenty four of these “AChE membranes” can be simultaneously arranged into the 24 wells (of about 5 mL of volume) of a plate to make sequential incubation and parallel inhibition of the enzyme allowing the successive measurements of the enzymatic activity. Three out of the 24 wells are schematically represented in Fig. 1: the membranes, fixed on a needle, can be inserted in and removed from the solutions at a fixed time during the inhibition, the washing and the incubation steps.

Figure 1. The membranes, fixed on a needle, can be inserted in and removed from the solutions at a fixed time during the procedural steps. Three out of the 24 wells are schematically represented. In the figure, a membrane is applied on the grape and dropped with PB, as described in the text.
In Fig. 1, a picture of the direct application of the membrane on a grape for the detection of cholinesterase inhibitors is showed (analytical details are further described).

2.2.2. Physically Entrapped Yeasts in Nylon Membranes

Genetically modified yeasts, physically entrapped into membranes, were tested in order to reduce costs of analysis. The purified AChE was thus replaced with an engineered (*Kluyveromyces lactis*) yeast, which expresses AChE activity from rat into the cell wall. Nylon membranes (0.2 μm pore size) were used and engineered yeast cells immobilised by syringe filtration of 1 mL cell suspension corresponding to 75 nU of AChE.

2.2.3. Immobilisation of ChOx on SPEs

ChOx in PB solution was immobilised onto the WE of a SPE with both PAP solution and a selective cellulose acetate membrane (MWCO = 100 Da), by dropping directly 10 μL on it. A dialysis membrane (MWCO = 12,000 Da) was then placed and a pressure of 36 kg/cm² was finally applied onto the assembled SPE. A commercial H₂O₂ electrode was also used for comparison and prepared in a similar way, but in this case the membranes with the immobilised ChOx were fixed onto the electrode by a neoprene O-ring.

Figure 2(A) and (B) shows the calibration curves obtained with both the commercial and the SPE ChOx-based biosensors.

Immobilised AChE membranes always show different activities (in the range 10–50 nU) after the immobilisation of the enzyme (paragraph 2.2.1) and washing by sonication. Figure 3 shows the comparison of two sets of measurements made without the inhibition step. This experiment estimates the reproducibility of the AChE activity determination.

2.3. Analytical Procedure

The proposed procedure for the analysis of OPs (in this paper paraoxon was used), consists in three operating steps.

1. Paraoxon calibration solutions, in the concentration range 10–75 μg/L, were prepared. The nylon membranes, where AChE (pure enzyme or yeast) was previously immobilised (according to the procedure of 2.2.1), were placed into contact with calibration solutions. The inhibition effect was previously evaluated as a function of the exposure time, and the best analytical results were obtained with 30 min (at a pesticide concentration of 37 μg/L).
Figure 2. Calibration curves (95% confidence intervals) for the choline biosensors obtained (A) with the commercial hydrogen peroxide electrode, (B) with a SPE. Signals from the recorder are reported in the inset as an example.

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2. "Inhibited" membranes were then rinsed and incubated in $10^{-3}$ M AChCl, using the apparatus in Fig. 1 which enabled rapid and operations on 24 membranes at a time, thus lowering the time needed for each analysis.

3. The residual activity of AChE on each membrane was sequentially measured under flow condition by using a ChOx modified SPE.

After washing and incubation of a set of 24 membranes in $10^{-3}$ M AChCl, the choline concentration, and inversely the AChE inhibition, were measured with high reproducibility (within 4.5% including the sample preparation) by using ChOx modified SPEs under flow conditions.

The same procedure was used for the detection of the insecticide on grapes and the following experimental design was used. Grapes were sprayed with a paraoxon solution in ethanol solution and allowed to dry, simulating the

\[ y = (1.06 \pm 0.08) x + (-3 \pm 2); r^2 = 0.89; \text{ coefficient of variation (CV)} = \pm 7.5%; n = 1. \]
agricultural practice. The nylon membrane (immobilised AchE) was deposited
directly on the contaminated grape (see Fig. 1), and 10 μL PB were dropped
onto the membrane to enable the interaction of the pesticide on it. The
analytical procedure then follows according to steps 2 and 3.

3. RESULTS AND DISCUSSION

The comparison of data obtained in the determination of choline by
the SPE ChOx-based biosensors with those obtained by commercial type
amperometric sensor-based biosensors [Fig. 2(A) and (B)] gave interesting
results, encouraging the use of SPEs. Even if their signal is unquestionably
more “disturbed,” SPEs seems to offer better results both for LOD and repro-
ducibility. The further experiments were so essentially based on the use SPE
ChOx-based biosensors.

The other encouraging results were obtained in the test on the repro-
ducibility of the AChE activity determination in spite of the fact that the
prepared AChE (immobilised) membranes always show different activities
(in the range 10–50 mU). Figure 3 clearly shows that the unavoidable limited
reproducibility of the immobilisation procedure does not significantly affect
the AchE activity determination.

One of the main objectives of our work was the development of a simple
procedure for the detection of cholinesterase inhibitors on grapes, giving a
simple but effective tool to verify the absence of residues of anticholinesterasic
insecticides.

The reproducibility of our procedure, (previously described in paragraph
2,3) can be deduced from Fig. 4. Three grapes were spray coated with para-
oxon (37 μg/L in ethanol), allowed to dry and then each grape was sampled
with three different membranes. A good reproducibility [within 5%, Fig. 4(A)]
was obtained. A slightly higher pesticide concentration [+15%, Fig. 4(B)] at
the bottom of the grape with respect to the middle and the top of the same
grape due to the outer waterproofing layer (cuticle) of the skin, allowing
the nebulised solution of paraoxon to slide along the fruit from the top to the
bottom.

The chosen approach, even if it requires further efforts for its optimisation
and standardisation, seems to be quite promising for “in field” test aimed
to statistically evaluate the absence of residues of OP and other anticholines-
teric insecticides in fruits and vegetables.

The other objective of this article was the evaluation of the usage of
genetically modified yeasts physically entrapped into membranes, reducing
the costs of analysis. The purified AChE was thus replaced with engineered
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Figure 4. (A) Reproducibility of the measurement of the inhibition ($n = 3$, using three different membranes). (B) Inhibition effect of the sprayed insecticides at different heights (1, top; 2, middle; 3, bottom) on the same grape (three membranes).
(K. lactis) yeast, which expresses AChE activity from rat into the cell wall. Only the second step for evolving AChE activity to be measured with ChOx biosensor required to be changed with respect to the pure enzyme-based procedure. The engineered yeast required longer time with respect to the pure enzyme to evolve the same choline concentration, as reported in Fig. 5 (in this figure, choline production vs. incubation time is compared for pure enzyme and engineered yeast). This effect was probably due to the longer time occurred for diffusion of the substrate through the cell wall of the yeast. However, this does not significantly affect the analytical performance of the genetically modified yeasts biosensors, as can be seen from inhibition curves reported in Fig. 6. Again the inhibition is due to paraoxon deposited on grapes, and curves were obtained with the pure enzyme (upper curve) and the engineered yeast (lower curve)-based biosensors. The yeast-based biosensor gives similar results compared to the purified enzyme biosensor under similar experimental conditions and this make interesting their use, considering the reduction of the costs of analysis.

Figure 5. Choline production vs. incubation time curves obtained with the engineered yeast (lower curve) and the pure enzyme (upper curve).
4. CONCLUSIONS

The usage of simple and cheap “home made” SPEs was evaluated in comparison with the usage of commercial H\textsubscript{2}O\textsubscript{2} electrode in choline biosensors. The obtained results encourage the usage of SPEs.

An already experimented analytical protocol for the determination of OP insecticides in water samples, with biosensors based on the usage of pure enzyme, was successfully applied to the determination of residues of OP insecticides on grapes. This protocol allows short time of analysis (22 samples/hr with parallel operation on 24 membranes) and good LOD (0.1 \text{\mu}g/L for paraoxon). This procedure was then extended to a biosensing system based on a recombinant yeast with a genetically expressed AChE activity from rat, obtaining similar analytical results and, again, acceptable time of analysis (17 samples/hr with parallel operation on 24 membranes).

Engineered yeast coupled with SPEs could represent an economic way for an EWS for OP insecticides (and other anticholinesterasic compounds) detection. These characteristics could allow the diffusion of reusable, “in-field,”
sensitive, reliable, cheap and easy-to-use sensors for environmental analytical application.

ABBREVIATIONS

AChE Acetyl cholinesterase
AChCl Acetyicholine chloride
BchE Butyrylcholinesterase
ChOx Choline oxidase
CV Coefficient of variation
ELISA Enzyme linked immuno-sorbed assay
EWS Early warning systems
GA Glutaraldehyde
GC Gas chromatography
LC Liquid chromatography
ISFET Ion selective field effect transistor
LAPS Light addressable potentiometric sensors
ln(LD50) Natural logarithm of the lethal dose (amount of a given toxic substance eliciting a lethal effect in 50% of the test organisms)
LOD Limit of detection
MS Mass spectrometry
OP insecticides Organophosphorous insecticides
PAP Polyazetidine
PB Phosphate buffer [0.1 M (pH = 7.0)]
PBS Phosphate buffer saline [(NaCl 0.1 M)]
PVC Polyvinyl chloride
RE Reference electrode (Ag/AgCl)
SPE Screen printed electrode
TFT Thick film technology
WE Working electrode

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