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review

Biosensors Based on Cholinesterase for Detection of Pesticides

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Summary

This review article (112 references) surveys studies on cholinesterase-based biosensors for analysis of organophosphate and carbamate pesticides. The kinetic characteristics of acetylcholinesterase and butyrylcholinesterase enzymes are briefly discussed with respect to bioanalytical applications. Several methods for measurement of enzyme activity are introduced together with suitable transducers (potentiometric, amperometric, spectrophotometric and fluorimetric). The detection limits for paraoxon as the reference pesticide are compared for various techniques and the applications of biosensors on real samples are summarised.

Keywords: biosensor, cholinesterase-based biosensors, organophosphate and carbamate, pesticides

Introduction

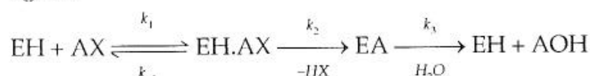
Fast, reliable and economical methods are strongly needed for detection of toxic compounds in the environment as public concern on the environmental pollution rapidly increases in recent years. The large number of analysed samples requires application of pre-screening methods, suitable for direct field use *(1)*. In this first step, the potentially hazardous samples could be identified and then further analysed in laboratory using more expensive and time-consuming classical analytical methods. Bioanalytical techniques could play quite an important role in this respect; a direct analysis is usually possible without any separation and cleanup steps, as the specific bioreagents could identify a single compound or a group of closely related analytes. Further improvement in the simplicity of analysis could be achieved by combination of the bioreagent as a recognition element with a classical chemical sensor – transducer, thus providing a biosensor. Some general requirements for such environmental biosensors were summarised *(2)*: low cost (1 to 15 US$ per analysis), portability, no external power supply, assay time 1 to 60 minutes, operation by minimally trained personnel and minimum sample preparation.

Besides immunochemical sensors utilising bioaffinity sensing principles, the inhibition of the biocatalytic activity of the enzymes cholinesterases is widely used for construction of biosensors for environmental applications. Acetylcholinesterase is the target site for two groups of widely used insecticides – organophosphates and carbamates; its inhibition disturbs the normal nervous function, finally resulting in the death of living organisms *(3)*. These insecticides are at present extensively used in agricultural and forestry industries, they are preferred for a relatively low persistency compared with e. g. organochlorine pesticides *(4,5)*. On the other hand, organophosphates and carbamates could possess high acute toxicity and the recommended protection interval should be carefully followed to prevent possible health problems. Besides environmental applications ChE-based sensing devices are also useful in military situations when the use of chemical warfare agents is expected. The use of nerve agents had been reported in 1991 during the Gulf War *(6)*, and quite recently, terrorists attacked the passengers with sarin at the Tokyo underground in 1995.

Characterisation of Cholinesterases

Two basic types of cholinesterase (ChE) exist: acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8). The physiological role of AChE in the nerve system is to terminate the nerve impulse transmission at cholinergic synapses; BChE (also known as pseudocholinesterase) is found in serum, its physiological role remains unknown. The amino acid sequences were determined for human BChE and for AChEs from *Torpedo californica* and *Drosophila melanogaster (7)*, the structure of the active site containing serine residue is also well known *(8)*. The hydrolysis of the natural substrate acetylcholine (AX) proceeds through formation of the enzyme – substrate complex (EH.AX), the choline molecule (HX) is released and the acetylated

enzyme (EA) is formed, which is rapidly hydrolysed to acetic acid (AOH) and the free enzyme (EH) is released again:

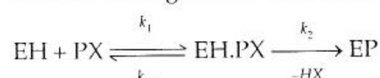


For reaction with substrates, expressions for the usual catalytic rate constant k_{cat} and the Michaelis constant K_M are given below:

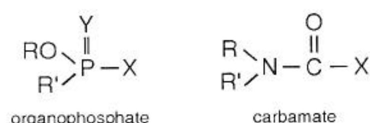
$$k_{cat} = k_{-1} + k_2/k_3 \qquad K_M = \frac{k_3(k_{-1} + k_2)}{k_1(k_3 + k_2)}$$

For AChE, the K_M values for acetylcholine, acetylthiocholine and ethyl acetate are 90 μmol/L, 57 μmol/L and 0.5 mol/L, respectively; the values of k_{cat} for acetylcholine is $7 \cdot 10^5$ min^{-1} and for ethyl acetate 10^5 min^{-1} *(9,10)*.

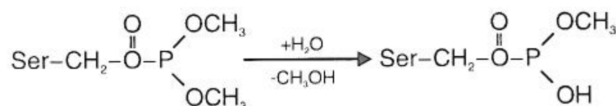
The reaction of cholinesterases with inhibitors organophosphates (PX) and carbamates is similar as with substrates, but the phosphorylated enzyme (EP) is quite stable resulting in the irreversible inhibition:



The general chemical structures of these two kinds of inhibitors are shown below:



In the case of organophosphates, the substituents could be as follows: R: alkyl, aryl; R': alkyl, aryl, alkyloxy, aryloxy or substituted amine residue; Y: either oxygen or sulphur; X: a »leaving group«, e. g. *p*-nitrophenyl, CN, F, phosphate diester. For carbamates, R and R': hydrogen, alkyl, aryl; X represents again the leaving group. In the reaction with cholinesterase, the leaving group is released and the serine residue in the active centre of the enzyme is substituted; thus, the phosphorylated (carbamylated) enzyme is formed which is catalytically inactive; the corresponding rate constant k_3 is $\approx 10^{-5}$ min^{-1}; for comparison, the acetylated AChE is hydrolysed quickly, $k_3 = 6 \cdot 10^5$ min^{-1}. The carbamylated cholinesterase is slowly hydrolysed spontaneously (k_3 = 0.012 min^{-1} for the $(CH_3)_2$N-CO-O-AChE complex *(11)*) so that in this case the inhibition is reversible. The phosphorylated AChE could be reactivated quite efficiently in the presence of certain nucleophiles. The most efficient reactivators are pyridine-2-aldoxime methiodide (2-PAM) *(12)* and 1,1'-trimethylenebis(pyridine-4-aldoxime) bromide (TMB_4) *(13)*. Even the covalent coimmobilisation of these agents together with ChE in the same polymer hydrogel was investigated *(14)*. In some situations, the spontaneous de-alkylation of one of the alkyl groups of *O,O*-dialkylphosphoserine occurs during the so-called »ageing« process:



The activity of the »aged« enzyme cannot be restored by reactivators *(15)*.

The kinetic rate equation for inhibition depends on the rate constant k_2 and on the dissociation constant K_D ($K_D = k_{-1}/k_1$) for the enzyme-inhibitor complex (EH.PX):

$$d[EP]/dt = k_2[EH.PX] = k_2[I]([E]_0 - [EP])/(K_D + [I])$$

Integration and rearrangement provides *(16)*

$$\Delta \ln S = k_2 t[I]/(K_D + [I])$$

For example, the values of K_D for complexes of paraoxon with AChE and BChE are 360 and 90 μmol/L. This equation could be further simplified provided that $[I] << K_D$, which is satisfied for most of the bioanalytical applications. Thus, the obtained simple equation *(17)* is widely used for characterisation of inhibiting properties using the bimolecular inhibition constant k_i:

$$\Delta \ln S = k_2 t[I]/K_D = k_i t[I]$$

The equation enables one to evaluate the concentration of anticholinesterase agents [I] using the measured decrease of ChE activity. It is also suitable for interpretation of measurements obtained either with free ChE or with biosensors. Usually, the initial activity (signal) S_0 is determined, the incubation with sample follows, and the residual activity S is measured. ($\Delta \ln S = \ln S_0 - \ln S$). Sometimes, the I_{50} parameter is used instead of k_i, but it could hardly serve for comparative purposes as I_{50} depends on the chosen incubation time, $I_{50} = \ln 2/(k_i\, t)$. The values of inhibition constants k_i are useful for preliminary characterisation of inhibiting properties for developed biosensors, as the differences in inhibiting properties resulting from the immobilisation of ChE are not large *(18)*. The examples of k_i for 55 various organophosphate and carbamate pesticides were published for cholinesterases from four different biological origins (AChE from electric eel and bovine erythrocytes, BChE from human and horse serums) *(19)*. Some examples of k_i values for insecticides as well as for nerve agents *(20)* are summarised in Table 1:

Table 1. Values of bimolecular inhibition constants k_i/mol^{-1} L s^{-1} for selected inhibitors of cholinesterases from various biological origins

	AChE electric eel	AChE bov. erythr.	BChE human serum	BChE horse serum
Organophosphates				
chlorfevinphos	$2.9 \cdot 10^4$	$9.0 \cdot 10^3$	$1.0 \cdot 10^6$	$2.1 \cdot 10^6$
dichlorvos	$4.2 \cdot 10^4$	$2.3 \cdot 10^4$	$2.3 \cdot 10^5$	$8.0 \cdot 10^4$
paraoxon	$2.2 \cdot 10^5$	$6.0 \cdot 10^5$	$1.5 \cdot 10^6$	$8.0 \cdot 10^5$
Carbamates				
aldicarb	$5.0 \cdot 10^4$	$1.3 \cdot 10^4$	$2.4 \cdot 10^4$	$1.0 \cdot 10^4$
carbaryl	$3.3 \cdot 10^4$	$1.8 \cdot 10^4$	$1.9 \cdot 10^3$	$7.0 \cdot 10^3$
carbofuran	$1.7 \cdot 10^6$	$8.0 \cdot 10^5$	$6.0 \cdot 10^3$	$3.1 \cdot 10^4$
Nerve agents				
sarin	$2.5 \cdot 10^7$	$6.3 \cdot 10^6$	$6.7 \cdot 10^6$	$1.2 \cdot 10^6$
soman	$1.0 \cdot 10^8$	$3.5 \cdot 10^7$	$1.9 \cdot 10^7$	$7.3 \cdot 10^6$

The bioanalytical measurement based on the decrease of enzyme activity after incubation with pesticides is for a long time used with soluble AChE *(21–26)*,

for a review, see *(27)*. The commercial kits are available either as a quantitative colorimetric test (Cholinesterase inhibition test, Boehringer Mannheim, Cat. No. 1 293 460) or as a qualitative/semi-quantitative version with visual evaluation (InQuest OP/Carbamate Screen, Ohmicron, Newtown, PA), the indicated detection limits are 0.05 and 30 μg/L, respectively. An improved manipulation could be achieved by immobilisation of ChE on a suitable supporting material *(28,29)*. To enhance inhibiting properties of some thionophosphates (parathion), the conversion to the more powerful inhibitors – oxones (paraoxon) is carried out by oxidation of samples using hydrogen peroxide *(30)*, bromine water or *N*-bromosuccinimide under milder conditions *(19)*. The inhibition by carbamates could be enhanced in the presence of suitable oximes *(31)*.

Construction of Biosensors

The biosensors are constructed by immobilisation of cholinesterase in a biocatalytic layer which is combined with a suitable transducer. The enzyme activity in the layer is quite important. Generally, for determination of inhibitors, the enzyme activity should be as small as possible to obtain high sensitivity and low detection limits *(32)*. Moreover, the performance of the biosensor should not be limited by the diffusion of substances *(33)*, otherwise the measured signal will not resemble the decrease of activity properly.

Two modes of analysis are possible with ChE-based biosensors. First, the preincubation approach is basically similar as for soluble ChE. The initial signal of the biosensor S_0 is determined; after washing, the incubation with the sample is carried out for a given time interval, and finally, the remaining signal S is determined. The inhibition I is calculated:

$$I/\% = 100\,(1 - S/S_0)$$

This approach is suitable for analysis of individual samples only. The dynamic (kinetic) operation of biosensors allows both characterisation of samples and continuous monitoring. The biosensor functions in the presence of substrate, and the continuous signal corresponds to the actual enzyme activity inside the biocatalytic layer. Any inhibition (due to the addition of samples or to the presence of inhibiting substances in the working environment) results in the decrease of activity which is followed by a decrease of signal. The rate of decrease (dS/dt) is proportional to the concentration of inhibitors. Improved results could be achieved when the relative inhibition RI is used *(34)*, which is calculated as the absolute rate corrected to the initial signal:

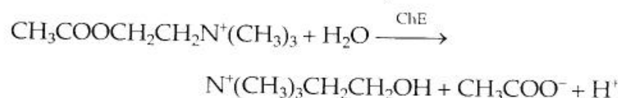
$$RI = (dS/dt)/S_0\,.$$

The relative inhibition now does not depend on the configuration of the biosensors and could be conveniently used to compare the sensitivities.

A large variety of construction designs of biosensors with cholinesterase were described, here especially those based on electrochemical and optical sensors will be addressed.

Electrochemical Biosensors

For a long time, pH sensors are used as transducers. Acetylcholine or butyrylcholine are used as substrates and acetic acid (butyric acid) released during enzyme hydrolysis causes the decrease of pH:

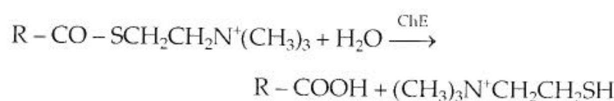


$$CH_3COOCH_2CH_2N^+(CH_3)_3 + H_2O \xrightarrow{ChE} N^+(CH_3)_3CH_2CH_2OH + CH_3COO^- + H^+$$

Usually, the glass pH electrodes are used, ChE could be immobilised by crosslinking with glutaraldehyde and albumin *(35,36)*, very thin membranes (50 μm) were prepared using activated acrylamide-based prepolymers *(37)*. By coating of the surface of glass electrode using AChE and crosslinking in glutaraldehyde aerosol, AChE layers only 2 μm thick were prepared which exhibited faster response *(38)*. Several other immobilisation methods were compared, and separate membranes prepared by crosslinking on a support were preferred *(39)*. A disposable enzyme layer was also prepared by covalent immobilisation of AChE on preactivated polyamide membranes *(40)*. pH electrode was combined also with the enzyme reactor made from glass beads coated with AChE *(41)*. The immobilisation of ChE directly on the glass pH electrode is less convenient; after several inhibitions, the biosensing layer should be either reactivated or replaced.

pH sensitive field effect transistors could be combined with ChE *(42,43)*, the enzyme could be crosslinked directly on the sensitive gate *(44,45)*. Another systems utilised immobilisation of biotinylated AChE through covalently bound streptavidine *(46)*, the light-addressable potentiometric sensors served as transducers *(47)*. A more reliable performance is possible with a differential ISFET *(48)*. An interesting system based on AChE incorporated in bilayer lipid membrane detected pH changes as alterations in the electrostatic fields resulting in ion currents transients *(49)*.

Several potentiometric ChE sensors are based on the measurement of the redox potential of thiocholine, which is generated by the enzyme hydrolysis from either acetyl- or butyrylthiocholine iodide *(50)*:



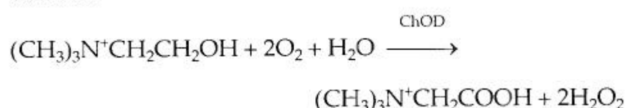
$$R-CO-SCH_2CH_2N^+(CH_3)_3 + H_2O \xrightarrow{ChE} R-COOH + (CH_3)_3N^+CH_2CH_2SH$$

The potential of the indicating and counter platinum electrodes is given by the thiocholine redox pair (0.2 V); after inhibition of ChE, less thiocholine is produced and the potential is determined by the I^-/I_2 pair (0.44 V). This principle was used for construction of the Continuous Aqueous Monitor (CAM, Midwest Research Institute, Kansas, USA) for detection of organophosphates *(51)*; BChE was entrapped in starch placed inside a poly(urethane) foam *(52)*; improved stability could be achieved using sorption on the gel of aluminium hydroxide *(53)*. This system is used also in the NAIAD detector (Nerve Agent Immobilised Enzyme Detector, Thorn EMI Simtec, Nottingham, UK) aimed for military applications. Both devices are rather robust but suitable for operation in field. Reoxidation of thiocholine with ferricyanide could

also be coupled with potentiometric detection *(54)*. The sulphide-selective electrode was employed as well *(55)*.

The benzoate-sensitive membrane sensor was used for potentiometric detection of BChE with benzoylcholine as substrate *(56)* and the butyrylcholine-sensitive potentiometric electrode was used as a transducer *(57)*.

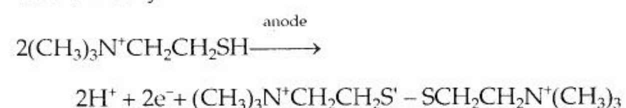
Two types of amperometric ChE biosensors are constructed. One possibility is to use choline oxidase (ChOD) for oxidation of choline produced in the ChE reaction:



$$(CH_3)_3N^+CH_2CH_2OH + 2O_2 + H_2O \xrightarrow{ChOD} (CH_3)_3N^+CH_2COOH + 2H_2O_2$$

The consumption of oxygen could be measured using Clark oxygen electrode *(58)*. Other methods employ the more sensitive oxidation of the produced hydrogen peroxide using platinum electrodes (0.65 V). All potentials in this review are referred *vs.* Ag/AgCl reference electrode *(59)*. Both ChE and ChOD could be coimmobilised in the same poly(vinylalcohol) layer made either on a supporting membrane *(60)* or on the surface of a carbon fibre *(61)*. The latter approach required rather high operating potential (1.2 V) for the oxidation of hydrogen peroxide on carbon. The coimmobilisation was performed also in gelatine *(62)* and acetylcellulose layers and by simple entrapment behind dialysis membrane *(63,64)*. To save ChOD after the change of inhibited enzyme layers, ChE free in solution could be used *(65)*. ChE/ChOD biosensors were constructed using conductive organic salt tetrathialfulvalene tetracyanoquinodimethane (TTF^+TCNQ^-) *(66)* which allowed to decrease the operating potential to 0.15 V. Both enzymes could be incorporated in a carbon paste electrode together with tetrathiafulvalene *(67)*. Disposable biosensors were produced using screen printed ruthenised graphite electrodes *(68)*.

Amperometric biosensors could operate with ChE only; when thiocholine esters are used as enzyme substrates, the produced thiocholine could be directly electrochemically oxidised:



$$2(CH_3)_3N^+CH_2CH_2SH \xrightarrow{anode} 2H^+ + 2e^- + (CH_3)_3N^+CH_2CH_2S' - SCH_2CH_2N^+(CH_3)_3$$

As suitable electrodes, either platinum (0.4 V) *(69)*, graphite *(70)* or graphite epoxy composite (0.7 V) with included AChE *(71)* were reported. The carbon composite electrodes modified with cobalt phthalocyanine *(34)*, tetracyanoquinodimethane *(72)* were prepared by screen printing and enabled oxidation of thiocholine at lower potentials. Tetrathiafulvalene and ferrocene were investigated as suitable modifiers *(73)* and the organic conducting salt microelectrode ($TCNQ^+TTF^-$) was also studied *(74)*. Production of thiocholine by ChE was determined voltammetrically using mercury-coated silver electrode *(75)* The amperometric version was developed later *(76)*. The other suitable substrate for ChE *p*-aminophenyl acetate provides *p*-aminophenol (PAP) during enzyme hydrolysis, and PAP could be conveniently oxidised (0.2 V) at platinum electrodes *(77)*. Similarly, indole produced from indolylacetate could be followed (0.35 V) *(78)*.

ChE sensor based on the thin film interdigitated array electrode operated on the conductometric principle *(79)*.

Optical Biosensors

For spectrophotometric measurement of ChE activity, the thiocholine esters could be used as substrates, thiocholine reacts with the Ellman's reagent *(80)* 5,5'-dithiobis(2-nitrobenzoic acid) and the resulting yellow colour is followed at 405 nm. This principle is used in the flow-through sensing system with automated exchange of ChE bound on magnetic beads in the enzyme reactor *(81)*. Other suitable substrate is α-naphthyl acetate, which is enzymatically hydrolysed and the released α-naphthol is then coupled to *p*-nitrobenzenediazonium fluoroborate (Fast Red GG), the resulting colour is measured at 500 nm. This was used in a flow-through system with AChE immobilised on controlled pore glass beads *(82)* and in the following work, this system was used as a specific detector in liquid chromatography *(83)*. AChE immobilised on Nylon beads was coupled also with plastic optical fibres. The colour change of the substrate resulting from enzyme hydrolysis was determined using the yellow LED as a light source and the system was used directly in field *(84)*.

Fluorometric sensors with ChE could employ either β-naphthyl acetate *(85)* or *N*-methylindoxylacetate *(86)* as fluorogenic substrates. The product of ChE reactions, thiocholine, could be sensitively detected after its reaction with *N*-(9-acridinyl)maleimide *(87)*. Portable fibre optic biosensors used fluorescein isothiocyanate (FITC) for fluorometric detection of pH changes resulting from hydrolysis of acetylcholine *(88)*. FITC could be immobilised in a dextran layer on the surface of the fibre *(89)*. For excitation, the evanescent field could be used in an optic fibre configuration *(90)*.

The three-enzyme chemiluminescent biosensor based on AChE, ChOD and peroxidase seems to be rather complicated. Hydrogen peroxide produced by ChOD is detected in the reaction with luminol *(91)*.

For comparison, the limits of detection for paraoxon achieved in various measuring configurations are shown in Table 2.

The comparable detection limits were obtained using either preincubation approach or kinetic measurements. The latter choice enables one to obtain results usually in less than 10 min. Moreover, kinetic analysis is also better suited for continuously measuring devices.

Some of the reported applications of ChE sensors for analysis of real samples are summarised in Table 3.

Conclusions

The advantage of ChE biosensors is that a wide group of similar compounds is detected with a single measuring device. Some approaches are possible for discrimination between organophosphates and carbamates. Kinetic behaviour of inhibited ChE is different for these two groups *(107)*. The inhibition of several different ChEs could provide more specific response pattern. On the contrary, coimmobilisation of several ChEs in the

Table 2. Limits of detection of paraoxon for various constructions and measuring approaches of ChE biosensors
INC n – preincubation for n minutes,
KIN – kinetic measurement.

Construction details	Procedure	γ(paraoxon)/μg L^{-1}	References
Glass pH electrode, regeneration, AChE in poly(acrylamide-methacrylate)	INC 60	0.3	*37*
pH sensitive ISFET, AChE crosslinked on the gate with glutaraldehyde	INC 10 INC 20	25 3	*48* *92*
Disposable modified graphite electrode, amperometric, BChE crosslinked with glutaraldehyde, AChE crosslinked with glutaraldehyde	INC 10 KIN KIN	0.85 0.08 88	*93* *93* *94*
Disposable interdigitated array electrode, conductometric, AChE crosslinked with glutaraldehyde	INC 10	270	*79*
Platinum electrode, amperometric, regeneration, AChE and ChOD coimmobilised in polyvinylalcohol	INC 30	2.0	*95*
ChOD crosslinked on platinum electrode, amperometric, AChE on a disposable Nylon membrane	INC 30	0.14	*96*
Flow analysis with Pt thick film sensor, AChE on magnetic particles, exchangeable reactor	INC 10	3	*97*
Spectrophotometric stopped-flow analysis, AChE on controlled pore glass beads, reactivation	KIN (FIA) INC 1	100 1.1	*82* *98*
Chemiluminescent flow sensor, fibre optic AChE, ChOD and peroxidase on methacrylate beads	INC 60	0.75	*91*
Fibre optic, spectrophotometric sensor, LED source, AChE on Nylon beads, reactor	KIN	0.27	*84*

Table 3. Examples of sample matrices for determination of anticholinesterase compounds

Sample	References
Surface and sea water	*97, 99, 100, 101, 105*
Drinking water	*81, 84, 97, 102*
Soil, sediments	*103, 104*
Fruit and vegetables	*105*
Meat	*106*

probability of a high response *(103)*. The inhibition of ChE could be due to the presence of pesticides, but the heavy metals present in the sample will contribute to the inhibition, too *(108,109)*. The effect of heavy metals could be removed if analysis of pesticides is performed in organic phase *(110)*. The extraction of pesticides from samples to organic solvents is usually the initial step of instrumental analysis. Such an extract could of course be analysed using a suitable ChE biosensor and an improved detection limit is achieved as a result of preconcentration *(111)*.

The interpretation of results obtained from ChE biosensors is not straightforward. Usually, the composition of the sample is unknown and consequently, the measured signal could not be used to determine the absolute concentration of a toxic compound. To solve this problem, the corresponding concentration of a »reference« pesticide (usually paraoxon) could be calculated. On the other hand, the signal could be interpreted as an anticholinesterase toxicity parameter and it will depend both on the concentration (c) of pesticides and on their inhibition properties (k_i). It was shown that an universal calibration curve for several individual pesticides was obtained using the dependence of inhibition on the ($k_i\,c$) parameter *(111)*. Many modifications of construction details (different kinds of ChE, immobilisation procedures, kinetic and preincubation measurements) could be employed, consequently, the comparison of results obtained in different working groups could be very difficult, if not impossible. Anyway, ChE biosensors are well suited for screening of large numbers of samples. In the case of positive identification, the more expensive and laborious instrumental methods should be used for validation *(112)*.

Significant research effort has been directed towards cholinesterase biosensors so that we can believe that these devices will soon become commercially available and will help to improve the protection of the environment as well as of human health.

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Biosenzori zasnovani na kolinesterazi za otkrivanje pesticida

Sažetak

Ovaj vrlo opsežan revijalni prikaz (112 referencija) obuhvaća radove o biosenzorima zasnovanim na kolinesterazi koji se primjenjuju za analizu organofosfornih i karbamatnih pesticida. Ukratko su razmatrane kinetičke značajke acetilkolinesteraze i butirilkolineresteraze u bioanalitičkoj primjeni. Navedeno je nekoliko postupaka za mjerenje enzimske aktivnosti uz prikladne transduktore (potenciometrijski, amperometrijski, spektrofotometrijski i fluorometrijski). Sažeto su prikazane granične vrijednosti određivanja paraoksona, kao referentnog pesticida, različitim postupcima te primjena biosenzora u stvarnim uzorcima.