analytical chemistry

ARTICLE

pubs.acs.org/ac

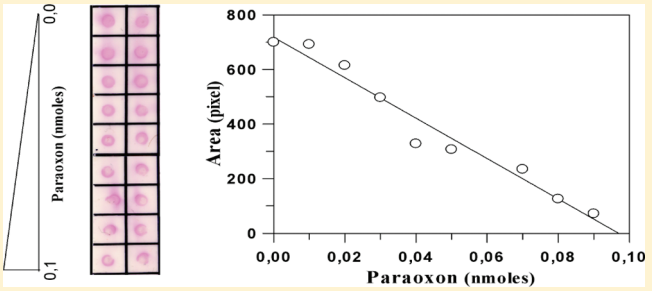
Thermostable Esterase 2 from *Alicyclobacillus acidocaldarius* as Biosensor for the Detection of Organophosphate Pesticides

Ferdinando Febbraio,* Luigia Merone, Giovanni Paolo Cetrangolo, Mosè Rossi, Roberto Nucci, and Giuseppe Manco

Istituto di Biochimica delle Proteine, CNR. Via P. Castellino 111, 80131 Naples, Italy

Supporting Information

ABSTRACT: Pesticides are the plague of modern times, although much needed in agriculture, causing damage to the entire ecosystem, including humans. The high operative costs and the requirement of specialized personnel for pesticide detection, incentive to develop alternative solutions such as the set up of cheap, rapid, and simple to use biosensors. In this work, we evaluate the possibility to use the esterase 2 from *Alicyclobacillus acidocaldarius* as a biosensor for the detection of specific organophosphate pesticides. With the recent demonstration of the very high affinity of esterase 2 toward paraoxon, a more complete analysis on the detection methods in water as well as in purposely contaminated fruit juices was carried out. The inhibitory effects of a wide range of other pesticides on esterase 2 were investigated, showing a better selectivity with respect to nonspecific reaction of acethylcholinesterases, the main target of organophosphate pesticides. The applied methodology allowed one to detect 2.75 × 10^{-3} ppm of neurotoxic agent, comparable to the efficiency of other acethylcholinesterase-based biosensors. Finally, a raw biosensor, based on EST2 immobilization on a nitrocellulose membrane, was devised and tested for paraoxon detection, showing longtime stability, reproducibility, and sensibility.



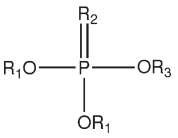
Frequently, in the last decades, the community has been facing the problems arising from the transfer of potentially harmful substances to the environment, altering the ecosystem, and to the human, causing pathological symptoms, and sometimes, if not often, death.[1−5] Nevertheless, some of these compounds, as the pesticides, are necessary and useful to the human well-being. Some actions have been undertaken; for example, this century has marked the disappearance in the environment of the highly persistent organochlorine pesticides, gradually replaced by organophosphates and carbamates that, in spite of a lower resistance to degradation, have become the most diffuse neurotoxic chemical compounds.[1] The mechanism of action of these neurotoxic compounds concerns mainly the irreversible inhibition of acetylcholinesterase,[6] a key enzyme for the correct activity of the nervous system. In particular, phosphorus-based compounds, known as organophosphates (OPs), are the most common and powerful acetylcholinesterase inhibitors, being designed to bind with high affinity to the active site of this enzyme.[6] The general structure of these molecules is summarized in Figure 1.

Because of toxicity to humans, the removal of excess of these compounds from the environment is mandatory, but a preliminary action of detection and monitoring is also required. At the present, the classical methods of pesticide detection makes use of gas chromatography (GC), high pressure liquid chromatography (HPLC), and recently mass spectrometry (MS) approaches.[7−10] These techniques are very powerful tools for monitoring toxic analytes, but they are expensive, time-consuming, and sometimes are not apt for in situ and real-time detection. In contrast, the easy handling of a pesticide biosensor would make possible the analysis by untrained people, also suggesting a probable diffusion in the domestic market.

Over the last decades, cholinesterase biosensors have emerged as ultra sensitive and rapid techniques for environmental monitoring and food quality controls.[11] These tools have the potential to complement or replace the classical analytical methods by simplifying or eliminating sample preparation protocols and making the testing in the field easier and faster with a significant decrease of the analysis costs. Unfortunately, most of the cholinesterase biosensors are not sufficiently robust to deal with raw samples and do not offer adequate selectivity. Some studies examined the possibility to use carboxylesterase activities in environmental monitoring.[12,13] This class of enzymes appears promising for employment in environmental monitoring with a number of organisms and testing scenarios, given the ease of activity assays and the stability in organic environments. Although it is still unclear if acetylcholinesterases or carboxylesterases are the most appropriate biomarkers, there are sufficient data to suggest that at the least further studies should be performed with carboxylesterases.[12,13]

In this work, we evaluate the possibility to use the esterase 2 from *Alicyclobacillus acidocaldarius* (EST2), a carboxylesterase belonging to the hormone sensitive lipase (HSL) family, as a

Received: July 30, 2010
Accepted: January 7, 2011
Published: February 02, 2011



R_1 =	$-CH_2CH_3$	$-CH_3$
R_2 =	O	S
R_3 =	alkyl, alkoxy, alkylthio, aryl, heterocyclic, aryloxy and arylthio groups	

Figure 1. General molecular formulas of organophosphates.

biosensor for the detection of specific organophosphate pesticides. EST2 is a monomeric protein of about 34 KDa, which hydrolyses monoacyl esters of different acyl chain lengths and different compounds of pharmacological and industrial interest.[14−16] The enzyme displays maximal activity on *p*-nitrophenyl (*p*NP) esters characterized by an acyl chain length of 6−8 carbon atoms, at an optimal temperature of 70 °C, although showing a discrete activity also at room temperature. Similarly to other enzymes from thermophilic organisms, EST2 shows long time stability in buffered solutions, appropriate resistance and activity at different pH values and temperatures,[17−19] and good stability in the presence of low concentrations of organic solvents, detergents, lipids, and so on.[20,21] The EST2 gene was cloned and expressed in the mesophilic bacteria *E. coli*;[14] the purification process was very easy using a thermo-precipitation step of the host proteins, yielding a high protein quantity in a short time and with low costs. The peculiarity of the carboxylesterases like EST2 to remain soluble in a medium containing a high concentration of molecules formed by chains or rings of carbon atoms makes it very simple to adsorb it in a quite irreversible way on carbon polymeric matrixes (e. g., nitrocellulose). Finally, the EST2 3D structure has been solved at 2.6 Å,[22,23] giving the possibility to have a model structure for the re-design of the active site.

Recently, we have demonstrated that EST2 is highly sensitive to the action of paraoxon, that is a specific acetylcholinesterase irreversible inhibitor, and in particular, EST2 seems to be endowed with a high affinity toward this OP pesticide.[24] Indeed, the EST2−paraoxon reaction cannot be described according to an usual pseudofirst-order kinetic as well as other irreversible inhibitions but is better described as a high affinity irreversible inhibition.

Thus, we tested the reproducibility of EST2 inhibition by paraoxon and the sensibility of assay and evaluated the possibility to use EST2 in real conditions on commercial products by assaying activity after paraoxon inhibition in fruit juices. Furthermore, we determined paraoxon concentrations in an enzyme-immobilized support like nitrocellulose, testing enzyme stability and assay sensibility.

The evaluation of the behavior of EST2 in the reaction with different OP compounds suggests a possible use of EST2 as the biocatalytic part of a multienzymatic biosensor for the selective detection of OP compounds in aqueous and organic solutions (liquid foods).

EXPERIMENTAL SECTION

Reagents. All reagents were of analytical grade and obtained from commercial sources. 2-[4-(2-Hydroxyethyl)-1-piperazino]-ethansulfonic acid (HEPES), diethyl-*p*-nitrophenyl phosphate (paraoxon), methyl-*p*-nitrophenyl phosphate (methyl-paraoxon), diethoxy-(4-nitrophenoxy)-sulfanylidenephosphorane (parathion), dimethoxy-(4-nitrophenoxy)-sulfanylidenephosphorane (methyl-parathion), 3-chloro-7-diethoxyphosphinothioyloxy-4-methyl-chromen-2-one (coumaphos), diethoxy-sulfanylidene-(3,5,6-trichloropyridin-2-yl)oxyphosphorane (dursban), diethoxy-(6-methyl-2-propan-2-ylpyrimidin-4-yl)oxy-sulfanylidenephosphorane (diazinon), diethoxy-(4-methylsulfinylphenoxy)-sulfanylidene-phosphorane (fensulfothion), Fast Blue RR salt, β-naphthyl acetate, and *p*-nitrophenyl hexanoate (*p*NP-C6) were from Sigma-Aldrich. The fruit juices were obtained from commercial sources and used before their expiration dates.

Enzyme Preparation. EST2 was overexpressed in the mesophilic host *E. coli* strain BL21 (DE3) and purified as previously described in Manco et al.[14] Purity was tested by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration was estimated by the optical absorbance at 280 nm, using a molar extinction coefficient of 1.34×10^5 M^{-1} cm^{-1} in 40 mM sodium phosphate buffer, pH 7.1, at 25 °C, as described in Manco et al.[25]

Enzyme Assay. The standard assay of esterase-catalyzed hydrolysis was carried out at 30 °C in 1.0 mL of reaction mixture containing 40 mM sodium phosphate buffer, pH 7.1, 4% (v/v) acetonitrile, and 100 μM *p*NP-C6 as substrate, monitoring the increase of absorbance at 405 nm due to release of 4-nitrophenolate (molar extinction coefficient of 13×10^3 M^{-1} cm^{-1}, at 405 nm) in a 1 cm path-length cell. Measurements were carried out in a double-beam Varian Cary 1E UV−visible spectrophotometer (Varian, Victoria, Australia), equipped for controlling temperature by the Peltier effect with an error of 0.1 °C, using an appropriate blank. An enzymatic unit was defined as the amount of enzyme that catalyzes the hydrolysis of 1 μmol of substrate in 1 min at 30 °C.

The esterase activity assay of immobilized EST2 was carried out by immersing the membrane in a filtered solution of Fast Blue RR salt (0.1 g) and 2-naphthyl acetate (0.1 g in 500 μL of methanol) in a 25 mL solution containing 25 mM Tris/HCl buffer, pH 8.5, 0.5 mM ethylenediaminetetraacetic acid (EDTA), and 0.25 mM $MgCl_2$. The esterase reaction develops 2-naphthol which couples to Fast Blue RR salt (a diazonium salt) forming a diazo dye complex, that being insoluble, permits one to detect esterase staining activity on the membrane;[26,27] the reaction was stopped by removing the substrate excess by repeated washing with H_2O. Colored membrane was digitally acquired by a 32 bit scanner, and spot intensity was determined by a densitometric software (Quantity One, BIO-RAD and Scion Image; Scion Corporation, Maryland (USA)).

Kinetic Constants. Enzyme kinetic constants for paraoxon inhibition were determined as described in Febbraio et al.,[24] using *p*NP-C6 as substrate in the concentration range of 0.5−200 μM. All measures were carried out at least three times, and the data was analyzed by the software Grafit 5.0 (Erithacus Software by R.J. Leatherbarrow) and QtiPlot 0.9.7.10 (Copyright 2004−2009 Ion Vasilief).

UV Spectroscopy. EST2 aliquots, 5 μM (5 nmoles) in 1 mL of 40 mM sodium phosphate buffer, pH 7.1, were used in UV spectroscopy measurements, before and after the addition of different paraoxon concentrations, in the range of 0.5−5 μM (0.5−5 nmoles). Measurements were carried out at 30 °C in a double-beam Varian Cary 1E UV−visible spectrophotometer (Varian, Victoria, Australia), equipped for controlling temperature by Peltier effect with an error of 0.1 °C, using a quartz cuvette of 1 cm optical path. Spectra were recorded in the wavelength range of 240−450 nm, with a scan speed of 50 nm/min, and a step resolution of 0.08 nm. A blank containing the buffer was used in all measurements.

Paraoxon Determination by Residual EST2 Activity in "in Vitro" Contaminated Fruit Juices. Increasing paraoxon

concentrations, from 10 to 150 μM, were added to 1 mL aliquots of fruit juices of different commercial origin and quality (peach, pear, etc.). The obtained solutions were centrifuged at 14 500 rpm for 5 min in an Epphendorf microcentrifuge, and the supernatants were recovered.

Supernatant aliquots of 1 μL (10 to 150 pmoles) were incubated in a 1 mL mixture, containing 100 μM (100 pmoles) of EST2 in 40 mM sodium phosphate buffer, pH 7.1, for a final juice concentration of 0.1%. Alternatively, similar experiments were carried out by adding increasing supernatant aliquots of the same paraoxon concentration in a 1 mL mixture, containing 100 μM (100 pmoles) of EST2 in 40 mM sodium phosphate buffer, pH 7.1, in order to obtain the same final inhibitor concentration, but with an increased final juice concentration in the range from 0.1 to 4%.

After few seconds from the paraoxon supernatant addition, aliquots of incubation mixtures were assayed along the time at 30 °C in the standard assay conditions. All the data were elaborated by the software Grafit 5.0 (Erithacus Software by R.J. Leatherbarrow) and QtiPlot 0.9.7.10 (Copyright 2004−2009 Ion Vasilief).

EST2 Immobilization and Paraoxon Determination on Membrane Assay. Immobilized enzyme was prepared by spotting aliquots of EST2, in 40 mM sodium phosphate buffer, pH 7.1, on a nitrocellulose membrane activated with nitric acid (HATF13250 50/PK, 0.45 μm white surfactant free HATF, Millipore), in delimited areas, and allowed to dry in order to optimize the immobilization process. After drying, the binding of protein with the membrane becomes very stable and is not observed to spread over time, and the enzyme activity is retained.

The determination of increasing paraoxon concentrations on enzyme-immobilized membrane was carried out using EST2 aliquots of 1 μL containing a constant concentration of 110 μM (110 pmoles) and, after immobilization, 1 μL aliquots containing different paraoxon concentrations in the range from 10 to 100 μM (10 - 100 pmoles) were added to each spot. Instead, the determination of a constant paraoxon concentration was carried out using EST2 aliquots of 0.2 μL containing concentrations in the range from 1 to 20 μM (1−20 pmoles), to which aliquots of 0.2 μL containing 5 pmoles of paraoxon for each spot were added.

After few seconds, in order to permit the complete absorption of solution on the membrane and the reaction between enzyme and pesticide, the residual activity of EST2 was determined as described in the enzyme assay on immobilized EST2.

RESULTS AND DISCUSSION

The evidence that carboxylesterase activities are sensible to the OP inhibition, dates back far to the 1970−80s, and was associated to the pesticide resistance in aphids[28,29] and mosquitoes,[30,31] but it is only recently that these enzymes have raised interest as part of biosensors in environmental monitoring.[12,13] We have focused our study on the EST2 from *A. acidocaldarius*, whose properties make feasible its use as the active part of a biosensor for paraoxon, one of the most used OP pesticide in agriculture, and a model compound in testing biosensing activities toward pesticides.[32] This OP is also widely studied for its neurotoxic, cancerous, and teratogenic effects,[33−35] which have been observed even at very low concentrations.[35,36] The compound is slowly degraded in the environment, allowing frequently its recovery in the food chain.[37]

As reported in Febbraio et al.,[24] the irreversible inactivation of EST2 by paraoxon could not be described as pseudofirst-order kinetics but as a very-high affinity inhibition kinetics; therefore, we could only calculate the apparent rate constants by continuously monitoring the enzymatic reaction in the presence of the inhibitor.[38] In agreement with previously published data,[24] the calculated parameters indicate an apparent rate constant of 17.52 min^{-1}, an apparent affinity constant of inhibition of 1.7 μM, and an inhibition specificity constant of 10.32 mM^{-1} min^{-1}. Interestingly, a survey of the literature seems to indicate that the apparent inhibitor specificity constant of EST2 is similar to, or higher than, those of other acetylcholinesterases from different sources and determined by the same approach.[38] In particular, the calculated values of apparent rate constant for paraoxon inhibition of electric eel,[39] bovine erythrocyte,[40] and narcine timelei[38] acetylcholinesterase were 19.8, 14.2, and 18 min^{-1}, respectively; however, translated into the specificity constant values, the results are lower with respect to EST2, being 3.3, 0.79, and 1.5 mM^{-1} min^{-1}, respectively. EST2 kinetic values are also comparable with results obtained from other sources but described by pseudofirst-order kinetics. In fact, similar values in the range from 7.0 to 20 mM^{-1} min^{-1} were obtained for several acetylcholinesterases from brain of neotropical fishes in the inhibition studies with methyl-paraoxon.[41] It is important to note that, for acetylcholinesterases from some neotropical fishes showing higher k_i values from 40−50 to 187.2 mM^{-1} min^{-1}, the affinity toward the acetylcholine substrate is 2−4 times lower than the other acetylcholinesterases, making results uncertain. Further results reported for other acetylcholinesterases, in particular from insects, and always described by a pseudofirst-order kinetics, showed very high k_i values,[42,43] about one thousand times higher than EST2 kinetic values measured by continuous monitoring of the enzymatic reaction in the presence of the inhibitor. However, if the EST2 k_i values could be determined by a pseudofirst-order kinetics, they would become similar to the kinetic values of insect acetylcholinesterases because the kinetic values of electric eel acetylcholinesterase determined by a treatment of pseudofirst-order kinetics are about one thousand times higher than reported above.[42,43]

These results are the starting point to begin a study on the possibility of detecting OP pesticides in solution using EST2. Then, some spectroscopic methodologies have been applied in order to quantify the paraoxon concentration binding EST2 in the inhibition process.

The direct monitoring of EST2 inhibition by paraoxon was possible by measuring the release of *p*NP from paraoxon hydrolysis, as the consequence of covalent binding of phosphoryl group to serine 155 in the catalytic site of EST2. The signal at 405 nm (maximum of absorbance for *p*NP) increased linearly with the addition of paraoxon (Figure 2) to a fixed EST2 concentration, up to a value corresponding to a 1:1 ratio; further addition of paraoxon did not produce any further increment in the signal. In spite of the low signal monitored, due to the poor level of *p*NP generated in the assay, however, a good linearity and a great reproducibility were achieved in the EST2 titration with paraoxon, in particular at lower pesticide concentrations. In fact, close to the stoichiometric enzyme−inhibitor ratio, at higher paraoxon concentration, EST2 concentration becomes limiting, giving very similar values of inhibition (Figure 2). Thus, this result makes it feasible to use this method for practical biosensing purposes in clear water solutions, while its application appears more complicated in complex solutions.

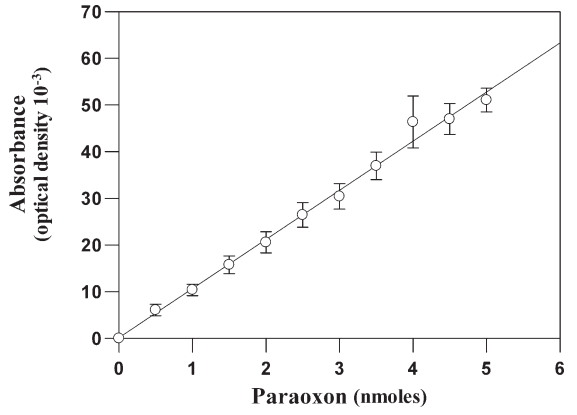


Figure 2. Absorbance increase at 405 nm of *p*-nitrophenolate, released by the paraoxon reaction with EST2, plotted against the paraoxon concentration. The values are normalized for the absorbance of EST2 in the absence of paraoxon.

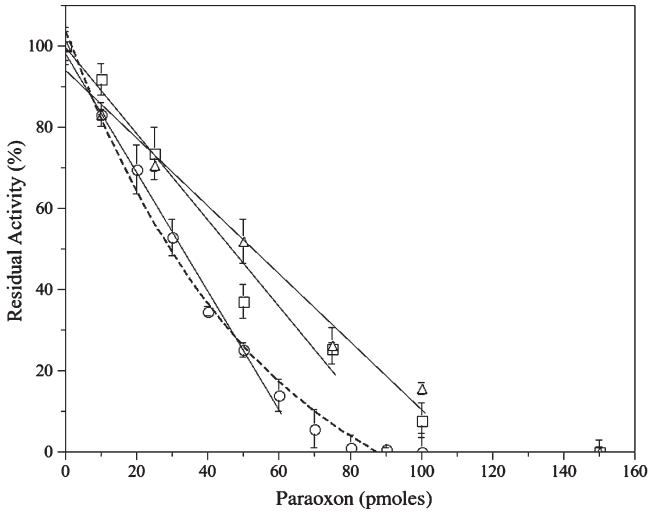


Figure 3. Residual enzyme activity in the presence of increasing paraoxon concentrations in sodium phosphate buffer, pH 7.1 (open circle), in the same buffer containing a constant (0.1%) concentration of centrifuged juice fruit serum (open square), and in the same buffer containing an increasing concentration (0−4%) of centrifuged juice fruit serum (open triangle).

The best result in the determination of unknown paraoxon concentration present in a solution was obtained by monitoring the EST2 residual activity after inactivation. Preliminary determinations of the residual esterase activity in the presence of very low paraoxon concentrations in the range of 10−80 nM (10−80 pmol/mL) revealed a good linearity in the curve of percentage of residual activity plotted against paraoxon concentration (Figure 3; open circle), allowing the determination of pmoles of paraoxon in solution. Addition to the solution of over stoichiometric concentrations of OP inhibitor with respect to EST2 concentration causes a complete inactivation of the enzyme. Although data were well fitted by a second degree equation with a coefficient of determination (R^2) of 0.98979 (curve in Figure 3; open circle), in the range from 10 to 60 pmoles of paraoxon, data were better described by a linear regression equation characterized by a strong value of R^2 of 0.992677 (straight line in Figure 3; open circle), suggesting that, as well as for spectroscopic measurements, greater linearity was observed at lower values of paraoxon far from stoichiometric ratio with the enzyme.

The remarkable stability of EST2 to organic solvents, detergents, and temperature[17−21] prompted us to test its use in a complex solution, such as fruit juices. Thus, in order to evaluate this possibility, we purposively contaminated aliquots of different fruit juices from commercial sources with paraoxon, clarified the serum by centrifugation to avoid interferences in the standard assay of the solid particles present in juice, and then tested the effect of these solutions on EST2 activity.

The EST2 activity was unaffected in the presence of serum concentrations up to 5% (data not shown); thus, we used concentrations in the range of 0.1−4%. The linear regression of residual activity in the presence of increasing paraoxon concentrations, but at a constant serum concentration of 0.1% (Figure 3; open square; $R^2 = 0.97130$), slightly diverged from the standard curve determined in water (Figure 3; open circle), indicating a probable protective effect of the complex molecular components present in the serum juice toward EST2 activity. In accordance, the same analysis, at increasing serum concentration (0 − 4%), relative to the addition of increased paraoxon concentration from the same mother solution to the enzyme, gave a slightly more divergent curve (Figure 3; open triangle; $R^2 = 0.98094$). Recently, it was observed that competitive inhibitors perform a protective effect on irreversible acethylcholinesterase inhibition,[44] so we could hypothesize similar effects exerted by some molecules present in the juices.

Nevertheless, the 10−20% deviation from the standard curve was observed only at higher paraoxon concentration while at lower concentrations divergence from values was less than 10%. Moreover the high reproducibility observed in the determination of residual activity in the presence of different kinds of juices, used at the same concentrations (data not shown), makes it feasible to construct a standard curve for paraoxon identification and quantization in liquid food.

These results make it important to define different calibration curves for the different samples analyzed, but at this time, we cannot exclude that a spontaneous degradation of the paraoxon happens in the presence of molecules present in the serum of fruit juices. This hypothesis could be confirmed by future GC/MS determinations, but it is possible that plots in the presence of fruit juices must be normalized to overlap data obtained in water, suggesting that we have measured in solution the real paraoxon concentrations that were lower than expected.

At the end, in order to realize a more simple tool to identify pesticides in solution, EST2 was rapidly and easily immobilized on the nitrocellulose sheet. The ability to bind to nitrocellulose is commonly accepted as being a universal property of proteins and has been widely used in many different fields of study. The exact mechanism by which proteins bind to nitrocellulose remains uncertain, although it is known that a number of forces are at work, specifically, hydrophobic interactions, hydrogen bonding, and electrostatic interactions. EST2 retains 100% of enzymatic activity after immobilization on nitrocellulose, measured as described in the Experimental Section.

In Figure 4, a brief description of the assay for the determination of paraoxon by immobilized EST2 was reported. After EST2 immobilization (Figure 4A), aliquots of paraoxon were added to immobilized-enzyme spots, in order to inhibit enzymatic activity. EST2 residual activity was quickly determined by incubating the membrane in the presence of 2-naphthyl acetate. This substrate is hydrolyzed by EST2 as described in Figure 4B, where the first

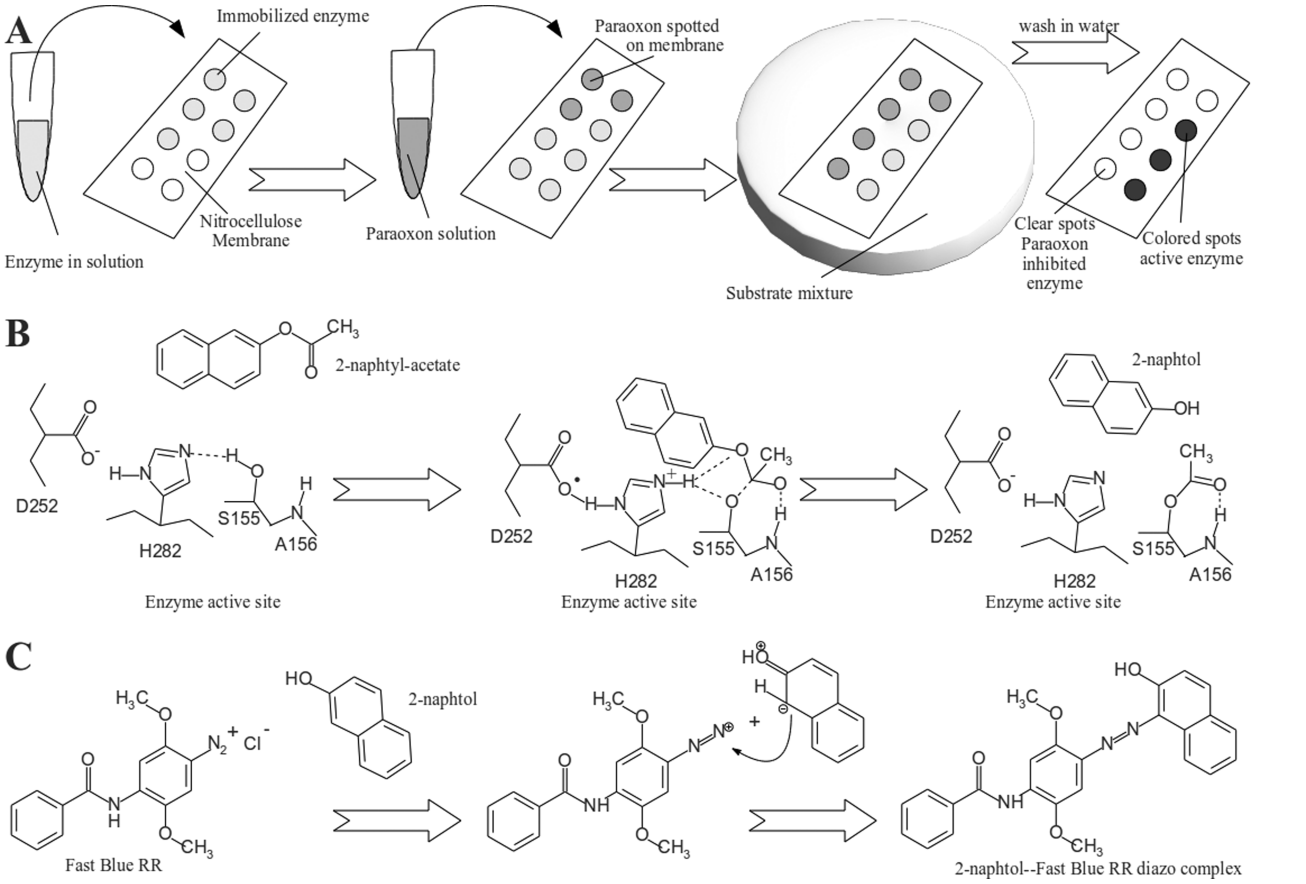


Figure 4. (A) Schematic representation of the EST2 immobilization process and on membrane assay of residual activity after paraoxon reaction. (B) Mechanism of reaction between EST2 and the 2-naphthyl acetate. See text for details. (C) Mechanism of coupling of 2-naphthol with Fast Blue RR salt (a diazonium salt) forming a diazo dye complex. The hydroxyl group in the 1 position of 2-naphthol is involved in a electrophilic substitution.

part of the reaction was reported. In particular, acylation of the enzyme results from the nucleophilic attack of the catalytic Ser155 on the ester and proceeds via formation of a tetrahedral intermediate (Figure 4B, center). Hydrogen bonds from the backbone amide groups of Ala156 stabilize the negatively charged tetrahedral intermediates occurring during the catalytic reaction. The reaction proceeds with the release of 2-naphthol and the formation of the covalent acylated intermediate (Figure 4B, right). Finally, the deacylation results from a nucleophilic attack of a water molecule on the acyl enzyme, again going through a tetrahedral intermediate (not shown).

The coupling reaction between 2-naphthol and Fast Blue RR diazonium salts (Figure 4C) involves an hydroxyl group in the 1 position of 2-naphthol by electrophilic substitution (the activating hydroxyl group is in the 2 position).[45] This reaction produces an insoluble and colored diazo complex that remains in the membrane grids.

After that, the membrane was washed extensively with top water and dried at room temperature. The residual enzymatic activity, determined as described in the Experimental Section, produces colored spots on the membrane whose saturation and spot areas vary with the different paraoxon concentrations, resulting in non-colored spots if this concentration equals or exceeds that of the enzyme.

The digital conversion of resulting membrane spots (Figure 5A), acquired by a 32 bit scanner, was analyzed by several types of software for densitometric calculations, as Scion Image (Scion

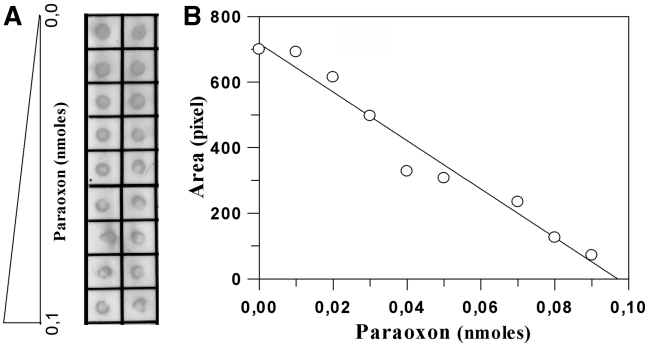


Figure 5. (A) Digital acquisition of nitrocellulose membrane after EST2 residual activity determination by Fast Blue and 2-naphthyl acetate. The spots containing the enzyme activity were colored in blue; the color intensity decreases with increasing inhibitor concentration. The concentration of paraoxon increases from top to bottom. (B) Plot of relative activity of immobilized EST2 inhibited by paraoxon against the inhibitor concentration in the range of 0−0.9 μM. On the *Y*-axis, the area in pixels determined for each spot is reported.

Corporation, Maryland (USA)) and Quantity One 1-D (BIO-RAD), in order to compare the results. The area in pixel relative to each spot, setting an appropriate value of color intensity, was plotted against the inhibitor concentration. As observed in Figure 5B, the results obtained fitted with a linear regression curve, suggesting the possibility to make a standard curve for unknown

dx.doi.org/10.1021/ac102025z |*Anal. Chem.* 2011, 83, 1530–1536

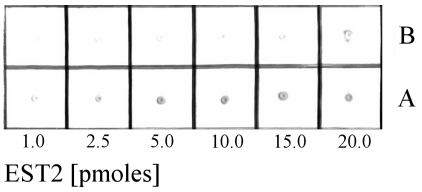


Figure 6. Digital acquisition of nitrocellulose membrane after EST2 residual activity determination by Fast Blue and 2-naphthyl acetate. The spots contain increased enzyme concentrations in the range from 1 to 20 pmoles (A). The concentration of paraoxon is 5 pmoles (B). The color in the spots, relative to the presence of residual enzymatic activity, disappears when inhibitor concentration is higher than enzyme concentration.

paraoxon concentrations, while the low range of identifiable concentrations was comparable to in solution assays.

Alternatively, we tested the possibility to obtain, with a good approximation, the paraoxon concentration in a solution by the immobilization on nitrocellulose membrane of increasing EST2 concentrations (Figure 6A). The addition of the same paraoxon concentration to the spots on the membrane that present different concentrations of enzyme results in an immediate quantitative determination of pesticide (Figure 6B). The first spot that shows residual activity indicates the threshold of paraoxon concentration present in the solution. The blue color present in the spot at 10 pmoles (gray in the Figure 6B) indicates a paraoxon concentration between 5 and 10 pmoles, with a resolution of 5 pmoles. However, having measured a minimal enzymatic activity of 1 pmole of EST2 (Figure 6A) by manual spotting, we do not exclude that using more precise automatic tools could be possible to further decrease this limit.

Studies on the stability of EST2 enzymatic activity, after immobilization on nitrocellulose membrane, were carried out storing enzyme immobilized membranes for 60 days (2 months) at RT or alternatively at 4 °C, with any particular attention to other environmental conditions except for the atmospheric dust. Activity assays at different times have indicated a complete recovery of EST2 activity all over the 2 months of monitoring. These data support the possibility to build simple and cheap biosensors using immobilized EST2 for a colorimetric detection of paraoxon.

Additional advantages in the use of EST2 are principally related to the higher selectivity of this enzyme. We have tested the EST2 sensibility to different pesticides in the presence of several OP inhibitors in a concentration ratio of enzyme/inhibitor up to 1:300 (Table 1 in Supporting Information), by monitoring the decrease of enzymatic activity along the time in the standard conditions. The EST2 activity was not affected at all by the OP inhibitors, parathion, methyl parathion, dursban, coumaphos, fensulfothion, and diazinon (Table 1 in Supporting Information), remaining unaltered (100%) after several hours of incubation. Also, EST2 activity assays in the presence of increasing concentrations of these OP inhibitors gave the same results. These observations indicate that these molecules do not react with EST2 as irreversible or reversible (competitive) inhibitors. Other analyses made to explore the possibility of a degradation of these compounds by EST2, through chemical or enzymatic hydrolysis, allowed us to exclude that the tested OP inhibitors are substrates for the enzyme (data not shown).

A careful analysis of the different molecular structures of the investigated inhibitors suggested that the presence of a heterocyclic ring or groups different from NO_2 in the benzoic ring of the compound makes its binding in the catalytic site unfeasible, very probably caused by a steric hindrance. In fact, the EST2 cavities in the active site present a different three-dimensional organization with respect to acetylcholinesterase ones, the latter being unspecifically inhibited by all OP inhibitors.[46,47] This is also indicated from the substrate volumes and typologies, small and simple for carboxylesterase with linear chains from three to six carbon atoms and bigger and complex for acetylcholinesterases with long chain and heterocyclic rings. The unsuccessful reaction between EST2 and parathion could be ascribed to the presence of a sulfur atom in place of the oxygen bound to the phosphorus atom. The reduced electronegativity and the increased radius of sulfur atom could decrease the phosphorus reactivity and the affinity for the binding site; besides, thiophosphoryl compounds are generally much less toxic than related phosphoryl derivatives. Different results were obtained for paraoxon and methyl-paraoxon, which irreversibly inactivated EST2 (Table 1 in Supporting Information) after a few seconds of incubation in the presence of the inhibitor. The very fast and stoichiometric reaction observed between EST2 and paraoxon (or methyl-paraoxon) suggested a high affinity toward these inhibitors.

CONCLUSIONS

The high affinity and specificity of EST2 toward paraoxon with respect to other OPs and carbamate pesticides[24] and the results obtained on pesticide detection by immobilized EST2 suggest that this enzyme could be used as an active part of a biosensor for the detection of this specific OP in the environment. Additionally, the high stability toward temperature, organic solvents, and pH allowed its use in extreme conditions, as in liquid foods. In conclusion, the selectivity toward specific OP in contrast with the broad range specificity showed by acethylcholinesterase allowed the qualitative and quantitative characterization of pesticides. This is the principal advantage in the use of EST2 or in general of esterases selectively sensible toward different OP. Furthermore, since these compounds are very similar in structure, it could be simple to obtain new EST2 mutants with different specificity for different OPs. This will make it possible in the future for the construction of multienzymatic biosensors for real-time, qualitative, and quantitative identification of a wide range of OPs.

ASSOCIATED CONTENT

Supporting Information. Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Tel.: +39 081 6132611. Fax +39 081 6132612. E-mail: f.febbraio@ibp.cnr.it.

ACKNOWLEDGMENT

This work was partly supported by a grant from Regione Campania Year 2000

REFERENCES

(1) Abdel Rasoul, G. M.; Abou Salem, M. E.; Mechael, A. A.; Hendy, O. M.; Rohlman, D. S.; Ismail, A. A. *Neurotoxicology* **2008**, *29*, 833.

(2) Durak, D.; Uzun, F. G.; Kalender, S.; Ogutcu, A.; Uzunhisarcikli, M.; Kalender, Y. *Environ. Toxicol.* **2009**, *24*, 235.
(3) Mink, P. J.; Adami, H. O.; Trichopoulos, D.; Britton, N. L.; Mandel, J. S. *Eur. J. Cancer Prev.* **2008**, *17*, 97.
(4) Salehi, F.; Turner, M. C.; Phillips, K. P.; Wigle, D. T.; Krewski, D.; Aronson, K. J. *J. Toxicol. Environ. Health, Part B: Crit. Rev.* **2008**, *11*, 276.
(5) Wigle, D. T.; Arbuckle, T. E.; Turner, M. C.; Bérubé, A.; Yang, Q.; Liu, S.; Krewski, D. *J. Toxicol. Environ. Health, Part B: Crit. Rev.* **2008**, *11*, 373.
(6) Bajgar, J. *Adv. Clin. Chem.* **2004**, *38*, 151.
(7) Galera, M. M.; García, M. D.; Valverde, R. S. *Talanta* **2008**, *76*, 815.
(8) Wang, L.; Liang, Y.; Jiang, X. *Bull. Environ. Contam. Toxicol.* **2008**, *81*, 377.
(9) Gervais, G.; Brosillon, S.; Laplanche, A.; Helen, C. *J. Chromatogr., A* **2008**, *1202*, 163.
(10) Hengel, M. J.; Miller, M. *J. Agric. Food Chem.* **2008**, *56*, 6851.
(11) Pohanka, M.; Musilek, K.; Kuca, K. *Curr. Med. Chem.* **2009**, *16*, 1790.
(12) Manco, G.; Nucci, R.; Febbraio, F. *Protein Pept. Lett.* **2009**, *16*, 1225.
(13) Wheelock, C. E.; Phillips, B. M.; Anderson, B. S.; Miller, J. L.; Miller, M. J.; Hammock, B. D. *Rev. Environ. Contam. Toxicol.* **2008**, *195*, 117.
(14) Manco, G.; Adinolfi, E.; Pisani, F. M.; Ottolina, G.; Carrea, G.; Rossi, M. *J. Biochem.* **1998**, *332*, 203.
(15) Manco, G.; Mandrich, L.; Rossi, M. *J. Biol. Chem.* **2001**, *276*, 37482.
(16) Manco, G.; Carrea, G.; Giosuè, E.; Ottolina, G.; Adamo, G.; Rossi, M. *Extremophiles* **2002**, *6*, 325.
(17) Foglia, F.; Mandrich, L.; Pezzullo, M.; Graziano, G.; Barone, G.; Rossi, M.; Manco, G.; Del Vecchio, P. *Biophys. Chem.* **2007**, *127*, 113.
(18) Mandrich, L.; Merone, L.; Pezzullo, M.; Cipolla, L.; Nicotra, F.; Rossi, M.; Manco, G. *J. Mol. Biol.* **2005**, *345*, 501.
(19) Mandrich, L.; Pezzullo, M.; Del Vecchio, P.; Barone, G.; Rossi, M.; Manco, G. *J. Mol. Biol.* **2004**, *335*, 357.
(20) Mandrich, L.; Manco, G.; Rossi, M.; Floris, E.; Jansen-van den Bosch, T.; Smit, G.; Wouters, J. A. *Appl. Environ. Microbiol.* **2006**, *72*, 3191.
(21) Del Vecchio, P.; Graziano, G.; Granata, V.; Barone, G.; Mandrich, L.; Rossi, M.; Manco, G. *Biochem. J.* **2002**, *367*, 857.
(22) De Simone, G.; Manco, G.; Galdiero, S.; Lombardi, A.; Rossi, M.; Pavone, V. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **1999**, *55*, 1348.
(23) De Simone, G.; Galdiero, S.; Manco, G.; Lang, D.; Rossi, M.; Pedone, C. *J. Mol. Biol.* **2000**, *303*, 761.
(24) Febbraio, F.; Esposito D'Andrea, S.; Mandrich, L.; Merone, L.; Rossi, M.; Nucci, R.; Manco, G. *Extremophiles* **2008**, *12*, 719.
(25) Manco, G.; Adinolfi, E.; Pisani, F. M.; Carratone, V; Rossi, M. *Protein Pept. Lett.* **1997**, *4*, 375.
(26) Miller, R. B.; Karn, R. C. J. *Biochem. Biophys. Methods* **1980**, *3*, 345.
(27) Kirkeby, S.; Moe, D. *Acta Histochem.* **1983**, *72*, 225.
(28) Devonshire, A. L. *Biochem. J.* **1975**, *149*, 463.
(29) Devonshire, A. L. *Biochem. J.* **1977**, *167*, 675.
(30) Mouchès, C.; Pasteur, N.; Bergé, J. B.; Hyrien, O.; Raymond, M.; de Saint Vincent, B. R.; de Silvestri, M.; Georghiou, G. P. *Mosq. Sci.* **1986**, *233*, 778.
(31) Raymond, M.; Beyssat-Arnaouty, V.; Sivasubramanian, N.; Mouchès, C.; Georghiou, G. P.; Pasteur, N. *Mosq. Biochem. Genet.* **1989**, *27*, 417.
(32) Zakir Hossain, S. M.; Luckham, R. E.; McFadden, M. J.; Brennan, J. D. *Anal. Chem.* **2009**, *81*, 9055.
(33) Barber, D. S.; Ehrich, M. *In Vitro Mol. Toxicol.* **2001**, *14*, 129.
(34) Carlson, K.; Jortner, B. S.; Ehrich, M. *Toxicol. Appl. Pharmacol.* **2000**, *168*, 102.
(35) Saleh, A. M.; Vijayasarathy, C.; Fernandez-Cabezudo, M.; Taleb, M.; Petroianu, G. J. *Appl. Toxicol.* **2003**, *23*, 23.
(36) Sun, X.; Liu, X. B.; Martinez, J. R.; Zhang, G. H. *Arch. Oral Biol.* **2000**, *45*, 621.
(37) Albero, B.; Sánchez-Brunete, C.; Tadeo, J. L. *J. Agric. Food. Chem.* **2003**, *51*, 6915.
(38) Liu, W.; Tsou, C. L. *Biochim. Biophys. Acta* **1986**, *870*, 185.
(39) Leytus, S. P.; Toledo, D. L.; Mangel, W. F. *Biochim. Biophys. Acta* **1984**, *788*, 74.
(40) Tsou, C. L. *Acta Biochim. Biophys. Sinica* **1965**, *5*, 409.
(41) Silva Filho, M. V.; Oliveira, M. M.; Salles, J. B.; Bastos, V. L.; Cassano, V. P.; Bastos, J. C. *Toxicol. Lett.* **2004**, *153*, 247.
(42) Nunes, G. S.; Montesinos, T.; de Oliveira Marques, P. R.; Fournier, D.; Marty, J. L. *Anal. Chim. Acta* **2001**, *434*, 1.
(43) de Oliveira Marques, P. R.; Nunes, G. S.; Rodrigues dos Santos, T. C.; Andreescu, S.; Marty, J. L. *Biosens. Bioelectron.* **2004**, *20*, 825.
(44) Eckert, S.; Eyer, P.; Mückter, H.; Worek, F. *Biochem. Pharmacol.* **2006**, *72*, 344.
(45) Augustinsson, K. B. *Biochim. Biophys. Acta* **1968**, *159*, 197.
(46) Skládal, P. *Anal. Chim. Acta* **1992**, *269*, 281.
(47) Worek, F.; Kirchner, T.; Bächer, M.; Szinicz, L. *Arch. Toxicol.* **1996**, *70*, 497.