Detoxification of Organophosphorus Compounds by Recombinant Carboxylesterase from an Insecticide-Resistant Mosquito and Oxime-Induced Amplification of Enzyme Activity

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ABSTRACT: Currently, bioremediation is a promising approach to the degradation of environmental pollutants. Here we describe the application of the recombinant insecticide-resistant mosquito carboxylesterase B1 to detoxify organophosphorous compounds. However, this approach has a major limitation: 1:1 stoichiometry of the enzyme detoxification of those organophosphorous compounds containing no carboxyl ester bonds, such as paraoxon, chlorpyrifos etc. To improve the effectiveness of the enzymatic detoxification of organophosphorous compounds, we used a combination of carboxylesterase B1 with the uncharged oxime diacetylmonoxime. It was demonstrated that the repeated addition of 20 times the molar concentration of paraoxon to carboxylesterase B1 every 2 h in the presence of 4 mM diacetylmonoxime did not result in significant inhibition of the enzyme. The stoichiometry of enzyme detoxification was higher than 45:1 and 20:1 for paraoxon and chlorpyrifos, respectively. The kinetic experiments on reactivation of organophosphorus compound–inhibited carboxylesterase B1 showed that the half-life for paraoxon- and chlorpyrifos-inhibited carboxylesterase reactivation is 0.75 and 0.88 h, respectively. Using the recombinant insecticide-resistant mosquito carboxylesterase with oxime is an effective approach for detoxification of organophosphorous compounds. Environ Toxicol 19: 154–159, 2004.

Keywords: detoxification; organophosphorous compounds; recombinant insecticide-resistant mosquito carboxylesterase; oxime; paraoxon; chlorpyrifos; diacetylmonoxime

INTRODUCTION

Organophosphorous (OP) compounds, which are known toxic substances, are used as pesticides, insecticides, and nerve gases (Donarski et al., 1989; Chapalamadugu and Chaudry, 1992; USDA, 1992). High-level exposure to these neurotoxins results in acetylcholine accumulation, possibly leading to death. Repeated or prolonged exposure can cause delayed cholinergic toxicity and neurotoxicity (Tuovinen et al., 1994). There is growing public concern about the accumulation of these pesticides in food products and water

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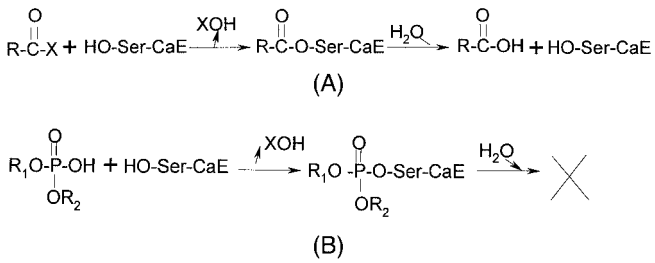


Fig. 1. (A) Hydrolysis of carboxyl esters by CaEs. (B) Inhibition of CaEs by organophosphorus compounds.

supplies, as well as a need to develop safe, convenient, and economically feasible methods for detoxification of these OP compounds.

With the wide use of pesticides for pest control, the ability of insects to survive these pesticides has required that insects evolve metabolic mechanisms for detoxification of a wide variety of compounds. Presently, more than 500 insect species have demonstrated resistance to pesticides, including the organochlorines, organophosphates, carbamates, pyrethroids, and newer Bt biopesticides (Sparks et al., 1993; Gould et al., 1995). Because the resistant insects can detoxify many kinds of pesticides in vivo, the enzymes encoded by the insects' resistance genes may also be useful in breaking down the pesticide pollutants in the environment. If large quantities of enzymes can be produced in a form that can be applied in the field, residues of the harmful chemicals may be able to be broken down (de Vries, 2000). In our laboratory it was shown that the organophosphate resistance of *Culex* is mainly conferred by the overproduction of esterase B1, which is the result of gene amplification. This gene has been cloned into *Escherichia coli* pET(28a) (Yan et al., 2000; Huang et al., 2001), which effectively expresses the carboxylesterase B1 (CaE B1) in a fermenter.

For OP compounds containing a carboxyl ester bond, such as malathion, enzyme hydrolysis by CaE B1 is based on the reversible acylation of a serine residues within the active center of the protein. This acylation causes the release of the alcohol moiety and of the corresponding covalently acylated enzyme. This acylated intermediate is hydrolyzed by the nucleophilic attack of water that releases the corresponding carboxylic acid moiety, plus the free active enzyme ready to initiate a new catalytic cycle (Fig. 1A). For those OP compounds containing no carboxyl ester bond, the first step is the phosphorylation of the enzyme. However, in this case, the phosphorylated enzyme cannot be reactivated by water, and it is impossible to regenerate the free active enzyme (Sogorb and Vilanova, 2002; Fig. 1B).

One possible approach to improving the enzymatic detoxification of OP compounds containing no carboxyl ester bonds by increasing the OP:enzyme stoichiometry is to use a combination of enzymes with a specific reactivator drug as a pretreatment. In general, OP-inhibited CaE can be reactivated rapidly by a selected oxime as long as it has not undergone aging (Caranto et al., 1994). In the present article, it is demonstrated that the addition of diacetylmonoxime (DAM) amplifies the effectiveness of CaE B1 in detoxifying OP. Thus, it may be possible to take advantage of the insect gene to bioremediate environmental pollution by OPs.

MATERIALS AND METHODS

Chemicals

Paraoxon, chlorpyrifos, methylparathion, and phoxim were used as supplied from domestic or foreign companies at >98% purity. The 2,3-butanedione monoxime (DAM, diacetylmonoxime) was purchased from Beijing Chemical Company (Beijing, China).

Cell Fraction and Enzyme Purification

The transformed *E. coli* BL21 (DE3) with plasmid pET-28a-B1 (Huang et al., 2001) was cultured in a 5-L fermenter (BIOTECH-2001, Shanghai, China). Bacterial cells were collected from the culture medium by centrifugation (5000g for 20 min). Cells were resuspended in 0.2 mol/L phosphate buffer (pH 7.0) and incubated with 50 mg/L lysozyme for 20 min at 30°C. The cells were then lysed using sonication, applying to each 10-mL volume 10 cycles of 15-s sonication, alternating with 45-s cooling periods. Nucleic acids and other cell debris were removed by centrifugation (12 000×g for 15 min). The supernatant was designated the "crude enzyme," which was then purified to electrophoretic homogeneity by metal chelation chromatography (data not shown).

Enzyme Analysis

CaE B1 activity was assayed with β-naphthyl acetate (β-NA) as the substrate (Asperen, 1962). The buffer system used was 0.2 mol/L phosphate buffer, pH 7.0. Substrate solutions were preequilibrated at 37°C. A 0.5-mL aliquot of diluted enzyme solution was added to a final volume of 3 mL. The samples were mixed, and the absorbance was measured in a Beckman DU-650 (USA) spectrophotometer at 555 nm.

CaE B1 Inhibition by Ops

Serial dilutions of OP were incubated with 0.1 μmol/L CaE at room temperature for at least 1 h in 0.2 mol/L phosphate buffer, pH 7.0. Residual CaE activity was measured as described above and plotted against the concentration of OP added to the reaction mixture to indicate the stoichiometry between CaE B1 and OP.

Detoxification of OPs by CaE B1 with DAM

The detoxification method of Caranto (1994) was used. The incubation mixture in 0.2 mol/L phosphate buffer, pH 7.0, contained 0.1 μmol/L CaE, 1–10 mmol/L DAM, and variable concentrations of OP compounds. Residual enzyme activity was determined after incubation at room temperature for different time intervals. In these studies two protocols were used. In the first protocol, various concentrations of OP were added to the reaction mixture at $t = 0$ to constitute different OP/CaE molar concentrations. After different time intervals, residual activity of the CaE was assayed. In the second protocol, equal amounts of OP were added to a series of reaction mixtures at $t = 0$. After 2 h the first reaction mixture was discarded after its residual activity was determined. To each of the remaining reaction mixtures the same amount of OP was added, and enzyme activity was determined in the second tube after another 2 h. This procedure was repeated numerous times until the enzyme activity decreased to 50% of its original value. Residue CaE activity was plotted against the cumulative concentration of OP added to each reaction mixture.

Kinetics of Reactivation of OP-Inhibited CaE B1

Inhibition of CaE B1 was performed in 0.2 mol/L phosphate buffer (pH 7.0) at 25°C with aqueous solutions of each OP. The CaE activity in the reaction mixture was measured after a 20-min incubation to confirm that greater than 90% inhibition of CaE had occurred. The OP-inhibited CaE was then separated from excess inhibitor by high-performance liquid chromatography (HPLC) on a Protein-Pak 300SW column (7.8 × 300 mm; Waters, Milford, Massachusetts) equilibrated with phosphate buffer. Each OP-inhibited CaE sample was compared to a corresponding control that had received identical incubation and chromatography treatments except for the absence of OP inhibitor. After chromatographic separation, the samples of OP-inhibited CaE were reactivated at 25°C by the addition of 4 mmol/L DAM in 0.2 mol/L phosphate buffer, pH 7.0. Samples of the reaction mixture were assayed sequentially for CaE activity at intervals up to 2 h. The observed rate constant for oxime reactivation of OP-inhibited CaE was calculated from the equation

$$v = V_{\max}(1 - e^{-\text{kobs}\, t})$$

where $V_{\max}$ is the maximal recovery of CaE activity and v is the activity at a given time (t). Because the reaction and aging of OP-inhibited enzyme are parallel first-order reactions, the true rate constants were calculated according to the method of Hovaned and Lieske (1972).

$$K_{\text{obs}} = k_r + k_a \text{ and } k_r/k_a = \%\ \text{reactivation}/(100\%\ \text{reactivation})$$

where % reaction is the maximal extent of oxime-induced reactivation of OP-inhibited CaE B1.

RESULTS

The presence of 4 mmol/L DAM affected the inhibition of CaE B1 by paraoxon (Fig. 2A). The experiment was carried out in 0.2 mol/L phosphate buffer, pH 7.0. The stoichiometry between paraoxon and CaE B1 was observed. Only 7% of the CaE activity was inhibited when greater than stoichiometric amounts of paraoxon were added to the mixture system containing DAM.

The reactivation of paraoxon-inhibited CaE was examined in the presence of 1, 2, 4, and 10 mmol/L DAM. The ratio of reactivation of paraoxon-inhibited CaE under the mentioned experimental conditions depended on the molar concentration of the oxime (Fig. 2B). In all cases more than 80% of the enzyme activity was restored after 4 h. The repeated addition of 20 times the molar concentration of paraoxon to CaE B1 in the presence of 4 mmol/L DAM every 2 h did not result in significant inhibition of the enzyme (Fig. 2C). A total of 2 nmol of paraoxon was detoxified by 0.1 nmol of CaE B1 in the presence of 4 mmol/L DAM. This conclusion was based on the observation that the addition of an aliquot of the last reaction mixture to the CaE B1 solution did not show any enzyme inhibition without DAM. The addition of a higher concentration of paraoxon to a reaction mixture containing a constant amount of CaE B1 in the presence of 4 mmol/L DAM and the determination of residual enzyme activity after 4 h showed that an increasing amount of enzyme was inhibited. More than 50% of the enzyme activity was inhibited when the total amount of paraoxon exceeded the molar concentration of the enzyme by a ratio greater than 45 (Fig. 2D).

To evaluate the kinetics of paraoxon neutralization by a single cycle of DAM reactivation, CaE B1 was inhibited by an excess of paraoxon, separated from paraoxon with HPLC, and reactivated with DAM. The apparent rate constant for reactivation by 4 mmol/L DAM was 0.92 h^{-1}. This rate constant suggests that under these experimental conditions, the half-life for CaE reactivation is 0.75 h and that DAM reactivation enables CaE B1 to detoxify one-half its molar equivalent of paraoxon every 0.75 h.

The presence of 4 mmol/L DAM also affected the inhibition of CaE B1 by chlorpyrifos (Fig. 3A). The addition of 0.125 nmol of chlorpyrifos to the reaction mixture containing 0.1 nmol of CaE completely inhibited enzyme activity in the absence of DAM. Under these experimental conditions, only 15% of the CaE B1 activity was inhibited in the presence of 4 mM DAM.

The amount of chlorpyrifos added to the reaction mixture was lowered to less than the amount required to inhibit all of the CaE B1. CaE in the presence of 4 mmol/L DAM continued to neutralize repetitive additions of chlorpyrifos

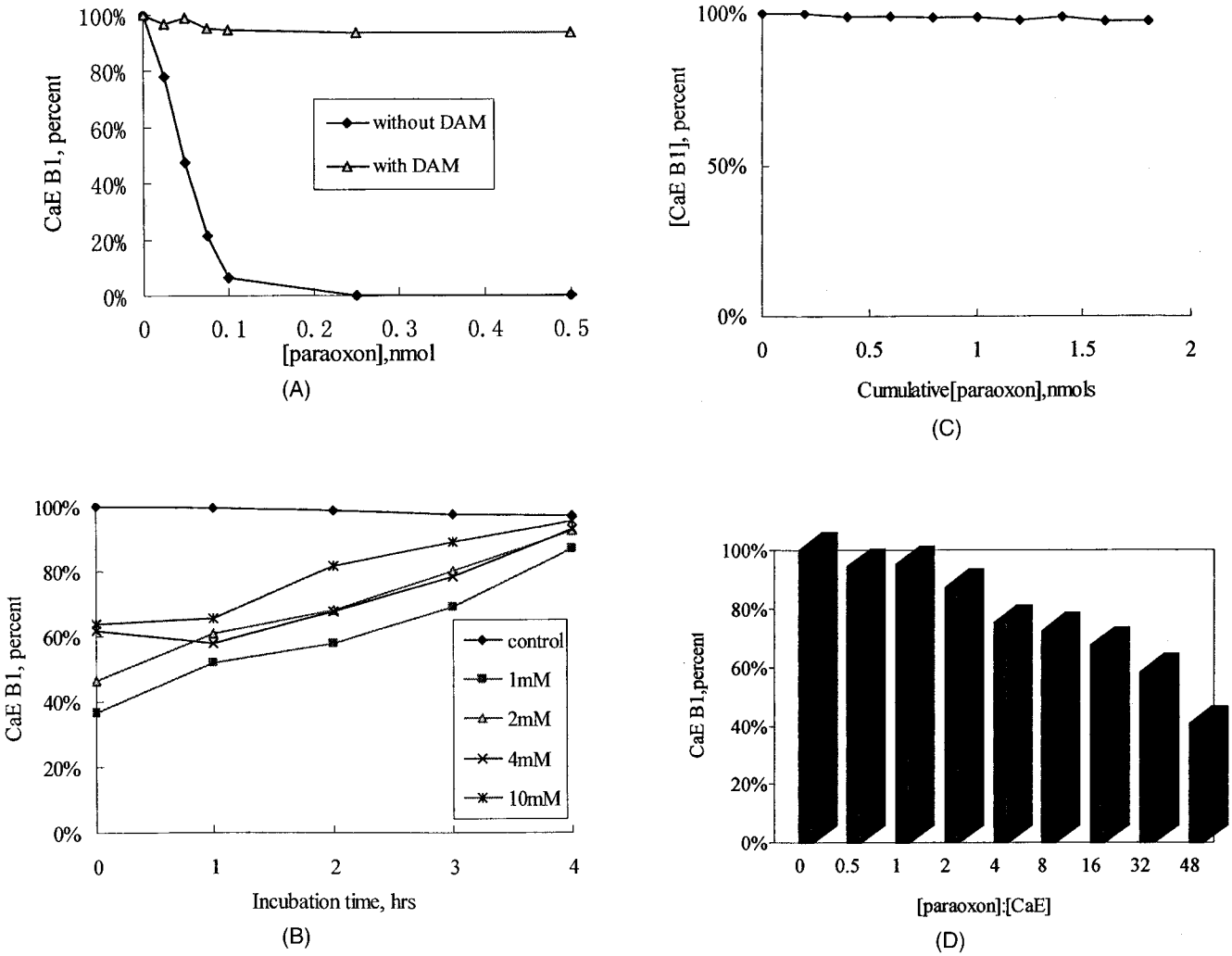
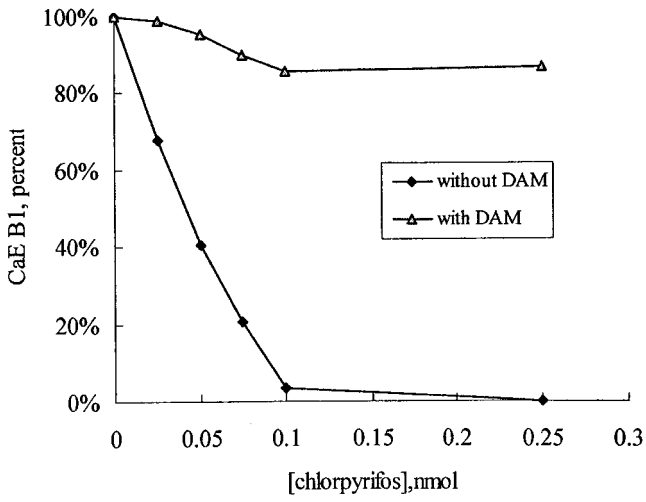


Fig. 2. (A) Inhibition of CaE B1 by paraoxon in the presence or absence of DAM. The incubation mixture contained 0.1 nmol CaE B1 and increasing amounts of paraoxon in a final volume of 1 mL (0.2 mM phosphate buffer, pH 7.0). The mixture was incubated at room temperature for 2 h; then residual CaE activity was measured. (B) Time course of reactivation of paraoxon-inhibited CaE B1 in the presence of various concentrations of DAM. The incubation mixture contained 0.1 nmol CaE B1, 1 nmol of paraoxon, and 1, 2, 4, or 10 mM DAM in a final volume of 1 mL (0.2 mol/L phosphate buffer, pH 7.0). The mixture was incubated at room temperature, and aliquots were assayed for residual CaE activity. (C) Reactivation of CaE in the presence of DAM after repeated additions of paraoxon. The reactivation of CaE B1 (0.1 nmol) by DAM (4 mmol/L) was carried out in the phosphate buffer (0.2 mol/L, pH 7.0) after repeated additions of paraoxon at 2-h intervals. The residual CaE B1 activity was measured 2 h after each addition of paraoxon. (D) Reactivation of paraoxon-inhibited CaE B1 by DAM. The incubation mixture contained 0.2 mol/L phosphate buffer, pH 7.0, 4 mmol/L DAM, 0.1 nmol CaE B1, and the indicated molar excess of paraoxon in a final volume of 1 mL. The mixture was incubated at room temperature for 4 h; then residual CaE activity was measured.

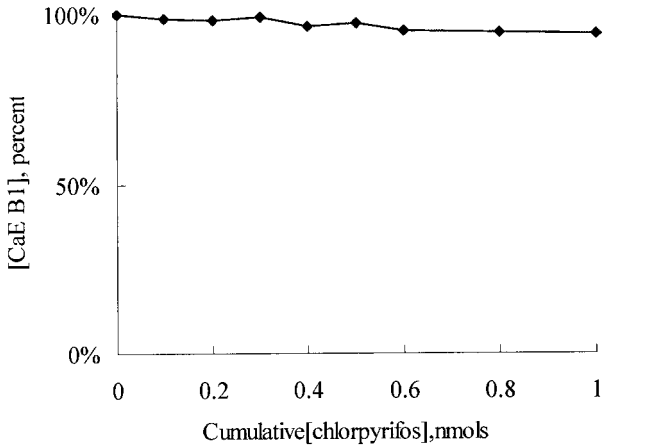
(Fig. 3B). A total of 1 nmol of chlorpyrifos was neutralized by 0.1 nmol of CaE B1 in the presence of 4 mmol/L DAM (approximately 10 times more than the expected amount). This continuous neutralization of chlorpyrifos was further evident when progressively larger than molar excesses of chlorpyrifos were added to CaE in the presence of DAM (Fig. 4C). When the total amount of chlorpyrifos exceeded the molar concentration of the enzyme by greater than 20, 60% of the enzyme activity was inhibited.

To study the kinetics of chlorpyrifos neutralization by a single cycle of DAM reactivation, CaE B1 was inhibited by an excess of chlorpyrifos, separated from free chlorpyrifos

on HPLC, and reactivated with DAM. The kinetics of DAM reactivation of chlorpyrifos-inhibited CaE differed from paraoxon-inhibited CaE B1 both in the extent of reactivation and the rate constant for reactivation. Finally, only 57% of the chlorpyrifos-inhibited CaE was reactivated, and the apparent rate constant for reactivation by 4 mmol/L DAM was 0.79 h^{-1}. This rate constant suggests that the half-life for CaE reactivation is 0.88 h and that DAM reactivation enables CaE B1 to detoxify one-half its molar amount of chlorpyrifos every 0.88 h.



(A)



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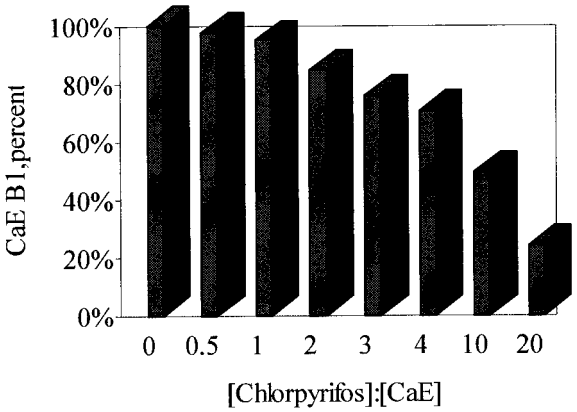

(C)

Fig. 3. (A) Inhibition of CaE B1 by chlorpyrifos in the presence or absence of DAM. The protocol described in the text was used. The incubation mixture contained 0.1 nmol CaE B1. (B) Reactivation of CaE in the presence of DAM after repeated additions of chlorpyrifos. The reactivation of CaE B1 (0.1 nmol) by DAM (4 mmol/L) was carried out in the phosphate buffer (0.2 mmol/L, pH 7.0) after repeated additions of chlorpyrifos at 2-h intervals. (C) Reactivation of chlorpyrifos-inhibited CaE B1 by DAM. The incubation mixture contained 0.2 mol/L phosphate buffer, pH 7.0, 4 mmol/L DAM, 0.1 nmol CaE B1, and the indicated molar excess of chlorpyrifos in a final volume of 1 mL. The mixture was incubated at room temperature for 4 h, and then residual CaE activity was measured.

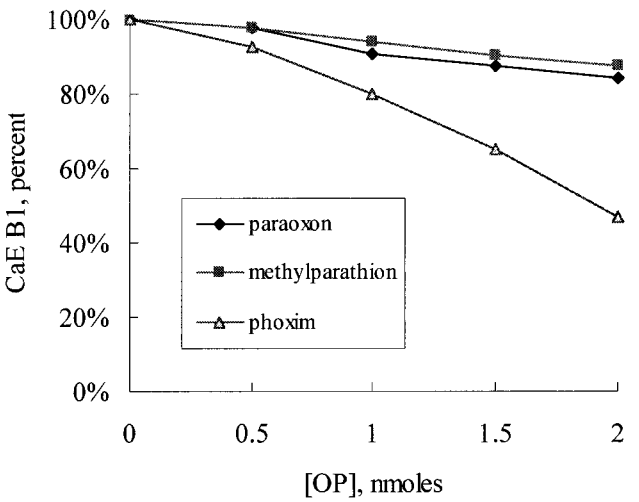

Fig. 4. Reactivation of various OP-inhibited CaE B1 by DAM. The incubation mixture contained 0.2 mol/L phosphate buffer, pH 7.0, 4 mmol/L DAM, 0.1 nmol CaE B1, and various concentrations of methylparathion, paraoxon, and phoxim. The mixture was incubated at room temperature for 4 h, and then residual CaE activity was measured.

The reactivation of methylparathion-inhibited, paraoxon-inhibited, and phoxim-inhibited CaE B1 by DAM was also examined (see Materials and Methods, protocol 1). The results demonstrated that after 4 h the effectiveness of DAM for reactivation of methylparathion and paraoxon was better than that for reactivation of phoxim (Fig. 4).

DISCUSSION

Enzymatic degradation of OP compounds has gained considerable attention during the past decade. However, most of the existing approaches to OP compound detoxification have not been implemented on large scale because of the

low degradation rate and high cost. So it is necessary to improve enzyme production and to amplify the effectiveness of enzyme detoxification. Presently, the combination of genetic engineering and microbial cultivation has become a powerful tool for producing recombinant gene products. The insecticide-resistance-associated esterase CaE B1 was produced on a large scale in our laboratory. In this study the results showed that the addition of DAM amplified the detoxification effectiveness of CaE B1 by continuously restoring its catalytic activity and thus detoxifying the OP.

Reactivation of paraoxon-inhibited and chlorpyrifos-inhibited CaE B1 by DAM showed that CaE was reactivated in the presence of the oxime DAM. The concentration of DAM required for such a reactivation was dependent on the rate of "aging" of the OP-inhibited CaE B1. Because the paraoxon-inhibited CaE "aged" relatively slowly ($t_{1/2}$ = 0.75 h), a large amount of paraoxon was neutralized continuously as long as the oxime concentration was maintained. This was not quite the same with chlorpyrifos-inhibited CaE because it "aged" more rapidly ($t_{1/2}$ = 0.88 h). The hydrolysis of OP compounds by CaE in the presence of DAM was confirmed by the test in which the addition of an aliquot of the end mixture to a fresh CaE solution without DAM produced no inhibition of CaE activity.

Previous studies had suggested that uncharged oximes, such as diacetylmonoxime, isonitrosoacetophenone, monoisonitrosoacetone, are effective reactivators of OP-inhibited CaE, whereas the most ineffective oximes—pyridine-2-aldoxime, pyridine-4-aldoxime—are all cationic oximes (Skrinjaric and Kralj, 1980; Wilson et al., 1992). In this work the results confirmed the above conclusion. This phenomenon is probably related to the differences in the substrate and inhibitor specificities of the enzyme. CaE has a broad specificity for uncharged substrates and inhibitors (Maxwell, 1992).

There may be useful applications of the recombinant insecticide-resistant mosquito carboxylesterase (CaE B1) with oximes (such as DAM) in many areas. It has the potential to be used for the decontamination of materials, equipment, and the environment. It is therefore expected that by using insect gene products, we can develop cost-effective, simple, efficient technology for detoxification of OP-contaminated environments.

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