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Bioremediation of Organophosphate Pollutants by a Genetically-Engineered Enzyme

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Microorganisms have been engineered to produce enzymes that can detoxify organophosphate contaminated water (Shimazu et al. 2001; Steven et al. 1990). Treating organophosphate pesticide (OPs) pollutants with the enzymes produced by genetically engineered organisms obviates the release of such organisms into the environment. Consequently, the development of enzyme-based treatment strategies is an increasingly important focus of environmental biotechnology (Steven et al. 1990).

There have been several studies of OP degradation in the field (Sreenivasulu and Aparna 2001, Adhya et al. 1987; Sharmila et al. 1988). In this paper, we evaluate the potential of a genetically-engineered enzyme to detoxify aqueous organophosphate wastes, specifically, parathion, malathion and monocrotophos.

MATERIALS AND METHODS

Malathion [S-1, 2-di (ethoxylcarbonyl) ethyl O,O-dimethyl phosphorothioate], Parathion (O-O diethyl O-4-nitrophenyl phosphorothioate) and monocrotophos (dimethyl 1-methyl-3-(methylamino)-3-oxo-1-propenyl phosphate) were obtained from the Qingdao Insecticide Company, China. Aqueous solutions were prepared by dissolving the above chemicals in sterilized, distilled water.

The carboxylesterase B1 gene was transferred to the *E. coli* plasmid pET28a (Qiao et al. 2000; Huang 2001). The *E. coli* BL-21 host was then transformed with the modified plasmid, pET28B1 (Figure 1). The transformed cells were observed to produce the detoxifying enzyme (Huang et al., 2001). In order to optimize enzyme production, and obtain larger quantities of enzyme, cells were cultured in a 5-L fermenter instead of flasks using an improved fermentation medium (Huang et al. 2001; Steven et al. 1990). Cells were harvested by centrifuging the cell suspension at 6000*g* and 4℃ for 15 min. 15mL of 0.02M

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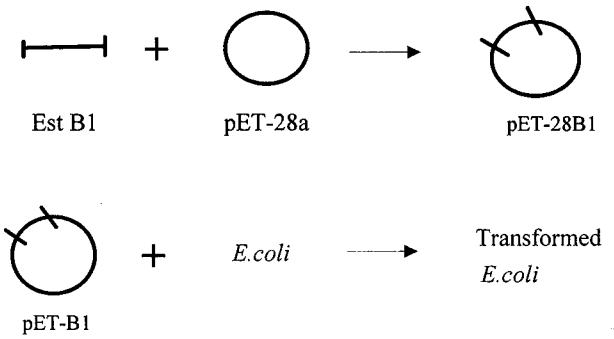

Figure 1. Schematic diagram of the genetic engineering used in this project. The carboxylesterase B1 gene was cloned into the pET28a plasmid to produce a new plasmid, pET28B1. This was then used to transform *E. coli* BL-21. (Adapted from Huang 2001)

phosphate buffer (pH 6.5) was then added to 5mg of cells and the resultant suspension well mixed, then 600μl lysozyme (10mg/mL) was added. The suspension was then incubated at 30 °C for 15min. The reaction was stopped by centrifuging the suspension at 6000*g* for 10min. The supernatant, or crude enzyme, contained 6mg/mL of detoxifying enzyme measured using the method described by Bradford(1976).

To study the temperature stability of the genetically-engineered enzyme,β-Naphthylacetate (β- NA) was used as a substrate to detect the enzyme's activity at different temperatures. Relative enzyme activity was assayed according to the method described in Asperen (1962). To study the effect of temperature on the degradation of β-NA, 6 mL portions of phosphate buffer (pH 7.0) containing 3×10^{-4} M β-NA and 3 mL crude enzyme were incubated at 28 °C, 34 °C and 40 °C. At regular intervals, three, 3 mL aliquot samples were taken from each flask and mixed with 0.5 mL diazoblue laurysulphate solution (DBLS). Variations in absorbance of the resultant degraded, red product were immediately estimated at 550 nm with a Beckman spectrophotometer. The effect of pH on β-NA degradation was determined by the hydrolysis of β-NA with crude enzyme adjusted to different pH levels (6.0, 6.5, 6.8, 7.0, 7.2, 7.5, 8.0) and incubated at 37°C. Each assay had a volume of 3 mL, consisting of 2.5 mL of the substrate solution in 0.1 M phosphate buffer at different pH. After reacting for 5 minutes, 0.5 mL DBLS was added and variations in absorbance were recorded at 550 nm with a Beckman spectrophotometer.

Degradation of the three pesticides was detected according to the method described in Leng and Qiao (1986). 15mL of sterilized, distilled water was added

to 5 mL of each pesticide, the resultant solution was mixed well and then 5mL of crude enzyme (about 30 mg protein) added. The mixtures were incubated at 34°C for different lengths of time (Figures 4-6). At regular intervals, three, 1 mL aliquot samples were taken from each flask to which either 1mL dichloromethane or petroleum ether were added. The samples were then desiccated with anhydrous sodium sulfate and the degradation products extracted with redistilled hexane and analyzed with a HP5890 gas chromatograph (GC).

The GC used was a HP-5890 Series II with a nitrogen phosphorous detector (NPD) on a fused silica capillary column (length 30m, 0.53 mm id x 0.5um film thickness, Supelco Corp. USA).

All three organophosphates were detected using nitrogen as the carrier gas at 1 mL/min. For Malathion and Parathion air pressure was: 58psi, hydrogen pressure: 21psi, nitrogen pressure: 52psi, air flux: 81.94mL/min, hydrogen flux: 3.2mL/min, nitrogen flux: 30.1mL/min. For monocrotophos, column flux was 2.7mL/min; air pressure: 58psi, hydrogen pressure: 21psi, nitrogen pressure: 42psi, air flux: 79mL/min, hydrogen flux: 3.0mL/min, nitrogen flux: 30.1mL/min.

In the case of Malathion, injector, column and detector temperatures were set at 250 ℃, 200 ℃, and 280℃, respectively and the minimum threshold of detection was 0.1 ng/μL. For Parathion, injector, column and detector temperatures were set at 250 ℃, 230 ℃, and 300℃ respectively and the minimum threshold of detection was 1 ng/μL. For monocrotophos, injector, column and detector temperatures were set at 250 ℃, 220 ℃, and 300℃ respectively and the minimum level of detection was 1 ng/μL.

RESULTS AND DISCUSSION

The use of enzymes to hydrolyze organophosphates has been considered by other workers (Steven et al. 1990). The enzymes used in this study were obtained directly from cells and were not purified prior to use. Such "crude enzymes" produced by transgenic bacteria must be stable over a sufficient period of time if they are to effectively detoxify pesticides in the field. We found that the performance of the crude enzymes tested in this study was temperature dependent with maximum activity occurring at 34-40℃ (Fig.2 and 3). Therefore, in order to maintain enzyme stability, we conducted pesticide degradation trials at 34℃. Optimum pH for crude enzyme preparations was 6.5-7.5 so we chose pH 7.0 as the standard for the degradation of the organophosphates. It should be noted that the detoxifying enzyme we produced has broad substrate specificity (Huang et al. 2001) and is therefore capable of hydrolyzing many related organophosphate,

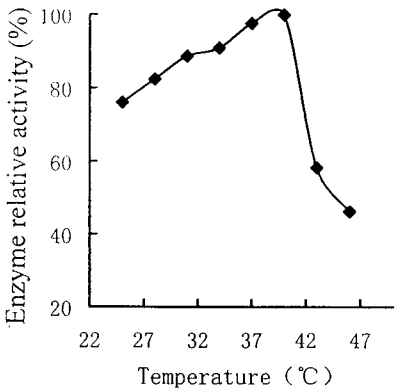

Figure 2. Effect of temperature on the degradation of β-NA

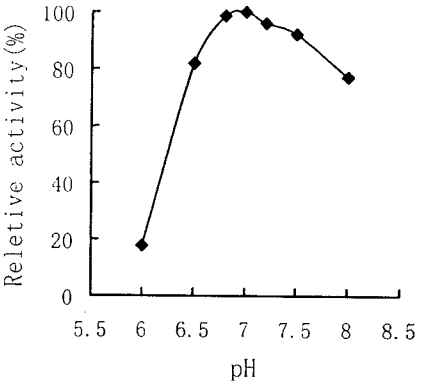

Figure 3. Optimum pH of detoxifying Enzyme

organochlorine and carbamate ester compounds. Consequently, we tested the ability of this enzyme to degrade a variety of compounds (malathion, parathion and monocrotophos) rather than just one. At 37℃, 70.5 % of malathion was degraded after 60 min and 79 % after 90 min (Fig. 4) compared to 83% of parathion after 6 hr (Fig. 5) and 13.4% of monocrotophos after 2 hr (Fig 6). These results show that our crude detoxifying enzyme degraded different kinds of pesticides containing ester bonds at different rates. Pesticides with carboxylester bonds (e.g. malathion and parathion) are typically degraded faster than those with other ester bonds (Huang et al. 2001). Our crude enzyme hydrolyzed malathion faster than the other two pesticides. Although the degradation rate of monocrotophos was relatively slow (Fig. 6), it may be possible to improve this by increasing enzyme quantity and/or decreasing the amount of monocrotophos.

Krieger and Dinoff (2000) determined malathion absorption and metabolite clearance rates among workers in date gardens. They found that each of the dimethyl phosphates (dimethylthio> dimethyldithio > dimethyl-) and both malathion mono- and diacids were present in urine as rapidly as 2-3 hr after exposure. On a micromole basis, dimethylthiophosphate and the malathion acids (MCA > DCA) were the most prominent metabolites in urine. This indicates that malathion is rapidly metabolized and excreted from the body in the urine. The order of ease of degradation in our study; malathion > parathion > monocrotophos is consistent with that of dimethylphosphates in general (dimethylthio> dimethyldithio > dimethyl phosphates).

Carboxylesterase is a collective term for the enzymes that hydrolyse carboxylic esters. The majority of OPs are esters of phosphoric acid and can therefore be

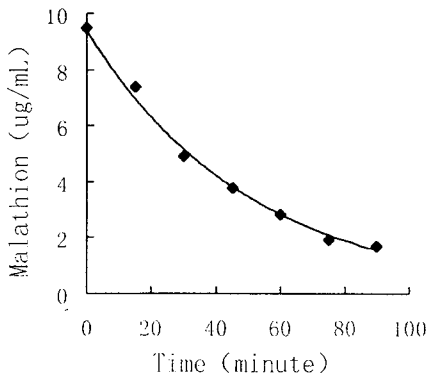

Figure 4. Degradation of malathion by detoxifying enzyme

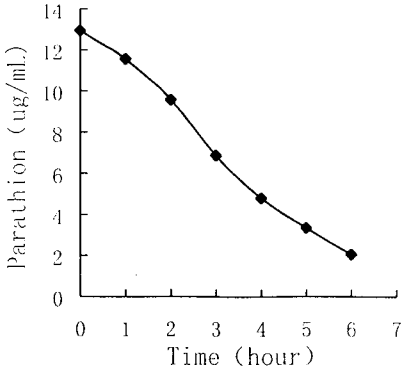

Figure 5. Degradation of parathion by detoxifying enzyme

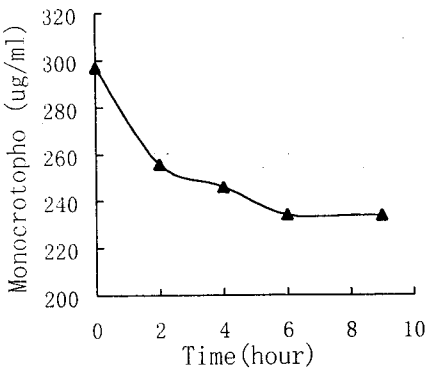

Figure 6. Degradation of monocrotophos by detoxifying enzyme

hydrolyzed by carboxlyesterases. Most OPs are administered in Phosphorothionate form, which is transformed into the organophosphate form by monooxygenases within the insect (Hemingway and Karunaratne 1998). The rate of interaction of these esterases with organophosphates is very fast. For example, in mosquitoes, the bimolecular rate constant for the reaction between Est B1 and parathion-oxon is 1.5 x 10^8 $M^{-1}min^{-1}$(Karunaratne et al. 1993). The deacylation rate is very slow, so it takes one molecule of enzyme about 32 h to hydrolyze a paraoxon molecule completely (Karunaratne et al. 1993). Deacylation rates are significantly higher for the aphid paraoxon inhibited esterase E4 which can completely hydrolyze a paraoxon molecule in 3h (Devonshire and Moores 1982). We obtained similar results using a genetically-engineered carboxylesterase to hydrolyze OPs (Figure 4-6).

Our results indicate that enzymes produced by genetically engineered organisms

organismshave considerable potential in reducing OP pollution in the environment.

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