

Decontamination of Vegetables Sprayed With Organophosphate Pesticides by Organophosphorus Hydrolase and Carboxylesterase (B1)

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Abstract

A genetically engineered *Escherichia coli* cell expressing both organophosphorus hydrolase (OPH) and carboxyl esterase (CaE) B1 intracellularly was constructed and cultivated. The harvested wet cells were vacuum dried, and the storage stability of the dried cell powder was determined in terms of OPH activity. Over a period of 5 mo, the dried cells showed no significant decrease in the activities of the detoxifying enzymes. The crude enzymes in 50 mM citrate-phosphate buffer (pH 8.0) were able to degrade approx 97% of the organophosphate pesticides sprayed on cabbage. The detoxification efficiency was superior to that of the treatments of water, detergent, and a commercially available enzyme product. Additionally, the products of pesticide hydrolysis generated by treatment with the enzyme extract were determined to be virtually nontoxic.

Index Entries: Organophosphorus hydrolase; organophosphates; carboxyl esterase B1.

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Introduction

Synthetic organophosphates (OPs) are widely used as pesticides in agriculture, municipal hygiene, and disease vector control. These compounds function by inhibiting the acetylcholinesterase in many living organisms, including humans. The repeated application of organophosphates in agriculture has increased the risk of contamination of groundwater and food products caused by these neurotoxic agents. Therefore, there is an urgent need to develop safe and economical methods for their detoxification.

Organophosphorus hydrolase (OPH), isolated from soil microorganisms from diverse geographic locations, has been shown to hydrolyze various organophosphates effectively (1). The hydrolase activities against the structurally different organophosphates were significantly different, however, ranging from the nearly diffusion-controlled limit for paraoxon to several orders of magnitude lower for phosphothioesters such as malathion. OPH variants with improved catalytic activity for several organophosphates such as chlorpyrifos, methyl parathion, s-demeton, and sarin, have been engineered using site-directed mutagenesis and in vitro-directed evolution (2). Many insect species resistant to organophosphates, including mosquitoes, have been found to possess the enzyme carboxyl esterase (CbE). Resistance to organophosphates in *Culex* has been identified to be primarily conferred by the overproduction of esterase B1, as a result of gene amplification. This gene has been cloned and a high-level expression of CbE B1 has been achieved in engineered *Escherichia coli* BL21 (3,4). CbE B1 exhibited a high degradation activity against organophosphates, such as malathion and chlorpyrifos (3,5).

Both purified OPH and genetically engineered microorganisms, including *E. coli*, expressing the enzyme intracellularly and on the cell surface, have been applied for detoxification of organophosphates such as parathion, methyl parathion, diazinon, and coumaphos (6,7). Although detoxification of organophosphates with immobilized enzymes and cell bioreactors has been well documented, there is no report of the detoxification of agricultural products such as vegetables based on a biologic strategy.

In the present study, we report the construction of a genetically engineered *E. coli* expressing both OPH and CbE B1 intracellularly, the long-term stability of enzyme activity in the lyophilized cell powder, and the application of the dry cell powder for detoxification of organophosphate residues on vegetables.

Materials and Methods

Chemicals

Parathion, malathion, parathion-methyl, phoxim, chlorpyrifos, and dichlorvos (DDVP) were obtained from Shandong Vicome Lunan Pesticides (Qingdao, Shandong, China). Dichloromethane, acetone, absolute ethanol, anhydrous sodium sulfate, and *p*-nitrophenol were purchased

from Beijing Chemical Reagents. Standard samples of malathion and DDVP were purchased from the National Research Center for Certified Reference Materials.

Bacterial Strains, Plasmid, and Culture Conditions

E. coli strain BL-21 (DE3) was used as expression host throughout the experiments. Plasmid pETDuet, used for coexpressing the OPH gene, and the CbE B1 gene were purchased from Novagen. Plasmids were maintained and propagated in *E. coli* DH5 α .

OPH and CbE B1 were coexpressed in *E. coli* BL-21 (DE3). The genetically engineered *E. coli* BL21 (DE3) harboring plasmid pETDuet-*opd*-b1 was constructed (8). Inoculum was prepared by transferring the cells into a culture tube containing 1 to 2 mL of Luria-Bertani medium supplemented with 50 μ g/mL of ampicillin. After incubating for 18 h, the culture was inoculated into 100 mL of the same medium in an Erlenmeyer flask. The preculture was subsequently used to inoculate 2.5 to 3 L of liquid medium in a 5-L fermentor (Aoxing Biotechnologies Co., Ltd., Shanghai, China) and grown at a controlled pH of 7.0 by adding ammonia until the OD₆₀₀ reached 0.5. At this time the expression of enzymes was induced by adding isopropyl- β -D-thiogalactoside (IPTG) and CoCl₂ to final concentrations of 1 mM, and the fermentation continued for an additional 24 h, at which time the cells were harvested from the culture medium by centrifuging at 5000g for 10 min. The wet cells were then dried at 20°C under vacuum, followed by freezing with liquid N₂ and grinding to a powder to release the enzymes from the cells.

Preparation of Enzyme Solution

Cell powder (0.12 g) was mixed with 1 mL of 0.2 M Na₂HPO₄-0.1 M citric acid buffer, pH 8.0, followed by centrifugation for 5 min at 12,000g to separate the cells. The supernatant was kept at 5°C for the activity assay and insecticide bioassay.

Electrophoresis

Expression of recombinant proteins was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a mini gel apparatus (MINI2-D; Bio-Rad, USA) using the Tris-glycine discontinuous system. Prepared protein samples were fractionated on 5% stacking and 8% resolving gels according to Leammli (9).

OPH Activity

The release of *p*-nitrophenol from 0.2 mM parathion in 10 min was used to measure organophosphate activity in phosphate-citric acid buffer, pH 8.0, at 25°C. To 2.92 mL of buffer, 60 μ L of 10 mM parathion preincubated at 25°C and 20 μ L of the supernatant were added, and the rate of change of absorbance was monitored using a Beckman DU800 spectrophotometer (Beckman Coulter, Co., USA). A solution without the enzyme extract was used as a negative control. Enzymatic activities were expressed as units (μ mol of parathion hydrolyzed/min) per g of cell powder.

CaE B1 Activity

CbE B1 activity was assayed with β -naphthyl acetate (β -NA) as substrate in 0.2 M phosphate buffer, pH 7.0, as follows: To 2.5 mL of 0.3 mM β -NA substrate solution preequilibrated at 37°C, 0.5 mL of diluted enzyme solution was added, and the mixture was incubated for 30 min followed by the addition of 0.5 mL of freshly prepared Diazoblue SDS reagent (0.3% fast blue salt in 3.5% aqueous SDS). The solution was incubated for an additional 15 min, and the color resulting from the formation of β -naphthol was measured at 555 nm. Enzymatic activity was expressed as units (defined as 1 μ mol of substrate hydrolyzed/min) per g of cell powder under the stated conditions (10).

Detoxification of Pesticides by CbE B1 and OPH

The test samples were prepared as follows: Ten grams of vegetable (cabbage) was sprayed with a known amount of pesticide followed by drying. The contaminated sample was suspended in 50 mL of enzyme or detergent solution (water for control) and incubated on an incubator shaker at 30°C and 150 rpm for 30 min. The vegetable was recovered and washed with water three times followed by drying at room temperature for 30 min. The vegetable leaves were then chopped into fine pieces and shaken vigorously with 30 g of anhydrous sodium sulfate and 0.2–0.8 g of activated carbon in a 250-mL glass vessel to dehydrate and remove the color. Next, the residual pesticides on the leaves were extracted with 50 mL of CH_2Cl_2 for 30 min at 200 rpm followed by filtration, drying, and reconstitution by acetone and, finally, they were analyzed by gas chromatography (GC).

An HP-5890 Series II gas chromatograph with a nitrogen phosphorous detector and a fused silica capillary column (30-m length, 0.53 mm id H 0.5 μ m film thickness; Supelco, USA) was used for analysis of the pesticides. Parathion was detected using nitrogen as a carrier gas at a flow rate of 30.1 mL/min. The air and hydrogen flow rates were 81.94 and 3.2 mL/min, respectively. The injector, column, and detector temperatures were set at 300, 230, and 310°C, respectively. The minimum level of detection for parathion was 1 ng/ μ L. GC conditions for analyzing the mixed pesticides were the same as for parathion except that the detector and injector were set at 300°C, and the column temperature was programmed from the starting value of 80°C for 3 min, to the final temperature of 200°C at a rate of 20°C/min and held for 8 min.

Bioassay

A mosquito larvae test was used to determine the detoxification efficiency and the toxicity of the degraded products following the method described by Raymond and Marquine (11). Depending on the number of available larvae, two doses and three replications per dose were used with each insecticide or enzyme products according to mosquito's LD_{50} . Organophosphate insecticides, parathion, malathion, and DDVP, were used in ethanol solutions.

Results and Discussion

For coexpression, two strategies may be used: (1) the target genes are cloned on two plasmids with two different antibiotic resistances (independent expression strategy) (12,13), or (2) the target genes are cloned in tandem in a transcription unit on a unique plasmid (single-operon strategy) (14,15). Although the use of two plasmids with the same origin of replication has been reported (16), most studies are performed with ColE1 derivatives combined with compatible plasmids bearing a p15A replicon. The former is a relatively simple process of DNA manipulation, but the copy numbers of two plasmids differ. The high copy number of the ColE1-based vectors would be likely to highly bias expression in favor of the genes carried by them and create an imbalanced protein expression in the cell. The latter seems to be complicated for constructing the coexpression strain; however, the relative amount of the produced protein is similar. Here we used Duet vector to coexpress OPH and CbE B1 simultaneously in *E. coli* BL21 (DE3) and expressed OPH and CbE B1 alone as control to determine whether the two enzymes from different species interact in one cell.

E. coli BL21 (DE3) is lysogenic for bacteriophage λ DE3, which contains the T7 bacteriophage gene (encoding T7 RNA polymerase) under the control of lac UV5 promoter. Two target genes of *opd* and *b1* are cloned downstream of T7 promoters of pETDuet plasmid. Coding regions of *opd* and *b1* are both controlled by T7 promoter in recombinant pETDuet-*opd-b1*. The addition of IPTG to the growing cell culture will induce expression of both OPH and CbE B1. The molecular mass of OPH and CbE B1 are about 35 and 65 kDa, respectively, as shown in Fig. 1.

Coexpression of CbE B1 and OPH in *E. coli*

To achieve high-level expression of CbE B1 and OPH in *E. coli*, the genetically engineered bacteria were cultured in fed-batch cultures. This culturing technique resulted in high cell densities and large amounts of recombinant proteins (data not shown). Gel electrophoresis of the crude extracts from cells on a 12% SDS-PAGE under reducing conditions followed by Coomassie blue staining showed bands at 65 and 35 kDa corresponding to the protein CbE B1 and OPH, respectively (Fig. 1).

Storage Stability

The activity of CbE B1 assayed in *E. coli* cell powder coexpressing OPH + CbE B1 was 1.754 U/g of dry cell powder, approximately the same as that expressing CbE B1 alone, about 1.514 U/g. Since this was much lower than the activity of OPH and because OPH is primarily responsible for the degradation of most organophosphates, we limited the focus of the present study to only to OPH.

The OPH activity of the cell powder was determined monthly, and the cell powder was stored at room temperature. As shown in Fig. 2, OPH activity was virtually unchanged until 5 mo. Additionally, once suspended

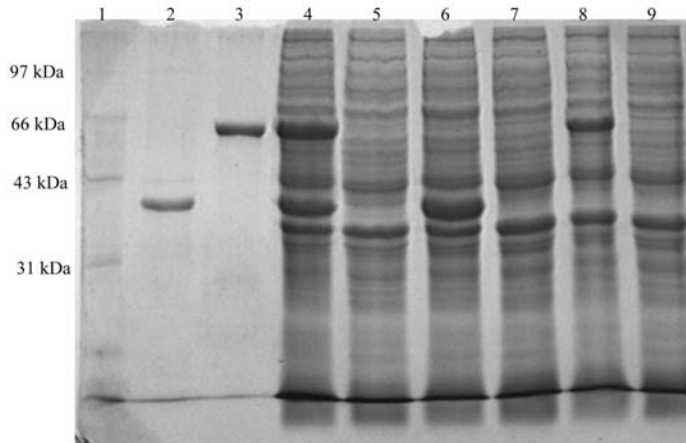


Fig. 1. Coexpression of CbE B1 and OPH in *E. coli*. Gel electrophoresis of the crude extracts from cells was performed. Lane 1, protein molecular weight standards; lane 2, purified OPH; lane 3, purified CbE B1; lane 4, expression of OPH and CbE B1 in *E. coli* carrying pETDuet-opd-b1; lane 5, whole cell lysates of uninduced *E. coli* carrying pETDuet-opd-b1; lane 6, expression of OPH in *E. coli* carrying pETDuet-opd; lane 7, whole cell lysates of uninduced *E. coli* carrying pETDuet-opd; lane 8, expression of B1 in *E. coli* carrying pETDuet-b1; lane 9, whole cell lysates of uninduced *E. coli* carrying pETDuet-b1. The molecular masses of standards are shown in the left margin.

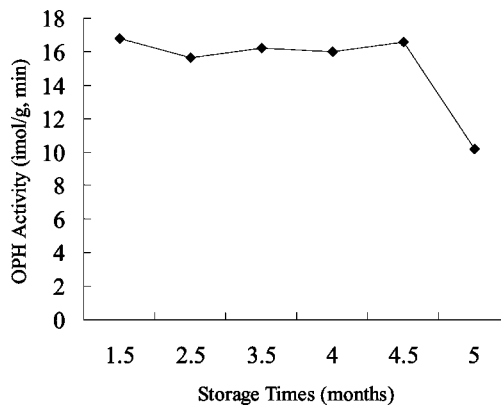


Fig. 2. Stability of OPH activity in cell powder when stored at room temperature.

in buffer, the suspension retained OPH activity for up to 1.5 mo at room temperature and 3 mo when stored at 4°C. Parathion (2.5 mg) was hydrolyzed by 0.05 g of cell powder in 10 min at 25°C. These results are in agreement with our previous reports of 1- to 1.5-mo stability of OPH in *E. coli* cells genetically engineered to express the enzyme on the cell surface. The excellent long-term stability of OPH in cells when stored either as dry powder or as liquid suspension at room temperature is very promising for application in detoxification of organophosphates.

Table 1
Efficiency of Coexpressed (OPH + CbE B1) Cell Powder Extract
and Other Products in Degrading Parathion-Contaminated Cabbage

Enzyme or detergent	Quantity of enzyme or detergent	Parathion (added) (mg)	Parathion residue (%)
Control	0 g	1.9	13.90
Product 1: Shuguoqing	60 μ L	1.9	12.80
Product 2: jiankangxiguan	0.1 g	1.9	8.00
Product 3: cell powder with acid + base	0.1 g (0.17 + 0.67)	1.9	6.22
Cell powder (OPH + CbE B1)	0.1 g	1.9	2.87

Detoxification of Pesticides

To evaluate the promise of OPH for detoxification of organophosphate pesticides, the enzyme extract of dried cells in pH 8.0 phosphate buffer was applied to degrade parathion, parathion-methyl, phoxim, chlorpyrifos, malathion, and DDVP sprayed on vegetables, and the degradation efficiency was compared with that achieved by washing the vegetables with the detergent shuguoqing (product 1) and enzyme jiankangxiguan (product 2) purchased from the market. As shown in Table 1, washing the parathion-contaminated vegetables with the enzyme extract of the cell powder with OPH + CbE B1 activities produced the best detoxification, ~97%, compared with 94–86% achieved by washing with commercially available enzyme product, detergent, or water. The addition of acid and base to the extract (in product 3) inhibited the activity of the OPH + CbE B1, probably owing to the loss of or decrease in the activity of the enzymes (details in Table 1).

As mentioned in the introduction, OPH has a broad substrate specificity and is able to degrade organophosphates with P-O (paraoxon, parathion, methyl parathion), P-F (DFP, soman, and sarin), and P-S (demeton-s, acephate, VX) bonds. We observed similar effective degradation of methyl parathion, phoxim, chlorpyrifos, and DDVP sprayed on vegetables when the extract of dried cell powder harboring OPH + CbE B1 enzymes was used in comparison with washing with water alone (Fig. 3). Although effective in degrading pesticides with P-O bonds, washing of the vegetables sprayed with the thiolester organophosphate insecticide malathion with the enzyme did not yield any improved degradation. This is in agreement with the observation of Lai et al. (17) that OPH has a very low catalytic activity for malathion ($k_{\text{cat}} = 0.0067 \text{ min}^{-1}$) and other P-S bonds containing organophosphates.

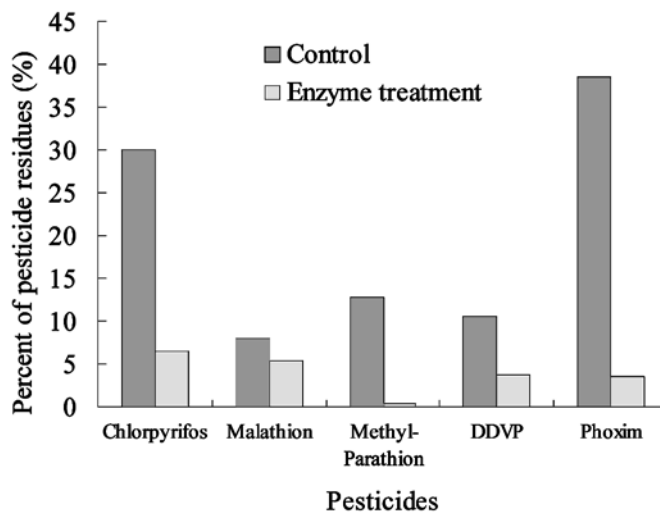


Fig. 3. Efficiency of removal of organophosphate residues on vegetables as percentage of amount sprayed when washed with water and enzyme solution.

Bioassays

To demonstrate further the efficacy of enzyme-based detoxification of organophosphates, we performed bioassays on Beijing Fengtai mosquitoes. The bioassay test involved determining the percentage of mosquito larvae surviving exposure to organophosphates before and after treatment with *E. coli* cell powder. As shown in Table 2, most of the larvae were killed on exposure to as low as 3 ppb of parathion. By contrast, >97% larvae were alive when exposed to a solution of 3 and 4 ppb of parathion treated with 24 and 30 mg of cell powder, respectively. Similar results were observed for the pesticide DDVP, with the survival percentage increasing from 43 to 100% for 300 ppb of DDVP treated with 24 mg of dry cell powder. The high percentage of larvae surviving exposure to hydrolyzed products suggests that these products were not toxic/poisonous and, hence, safe for pests.

As expected, there was no change in the survival percentage for malathion, because OPH has no effect on the degradation of this thiolester organophosphate pesticide.

Conclusion

The results presented in this study demonstrated that OPH and CbE B1 coexpressed in genetically engineered *E. coli* remained highly active for extended periods when stored as dried powder or as liquid suspension. The cell suspension was effective in decontaminating organophosphates sprayed on vegetables and the hydrolyzed products were not toxic.

Table 2
Bioassay of Mosquito Larvae Exposed
to Enzyme-Hydrolyzed Products of Pesticide

Organophosphate	Organophosphate concentration (ppm)	Cell powder (g)	Surviving larvae (%)	Surviving larvae (control) (%)
Parathion	0.004	0.024	76	0
Parathion	0.004	0.030	98	0
Parathion	0.003	0.024	97	9
Malathion	0.9	0.100	5	5
DDVP	0.3	0.024	100	43

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