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An Optical Resolution of Racemic Organophosphorous Esters by Phosphotriesterase-Catalyzing Hydrolysis

SHOKICHI OHUCHI, HIROYUKI NAKAMURA, HIROTO SUGIURA, MITSUAKI NARITA, AND KOJI SODE*

Department of Biotechnology, Tokyo University of Agriculture and Technology, 2-24-16 Nakamachi, Koganei, Tokyo 184, Japan

Index Entries: Phosphotriesterase; stereospecific reaction; organophosphorous insecticide; phosphotriesters; kinetic optical resolution.

INTRODUCTION

Commercially available organophosphorous insecticides, such as malathion, vamidothion, profenofos *(1)*, acephate *(2)*, and so forth, often contain chiral carbon, phosphorous, or sulfur atoms. These chiral insecticides are used as racemic mixtures because of the absence of effective resolution procedures. However, the insecticidal activity of the optically pure compound is different from the racemic compound. For example, ethyl 4-nitrophenyl phenylphosphonothioate (EPN), which contains a chiral phosphorous atom, is more toxic as the R-form (Rp-EPN) than as the S-form (Sp-EPN) or as the racemic mixture against mice, hens, and insects *(3)*. Therefore, further systematic analysis of biological activity of chiral insecticides is essential in order to design and develop novel insecticides with specificity. In addition, enantiospecific degradation of organophosphorous insecticides by soil bacterial enzymes should also be studied in detail for further understanding in selectively remaining enantiomers and their toxicity.

Phosphotriesterase (PTE) from native soil bacteria catalyzes the hydrolysis of organophosphate triesters and organophosphonate diesters (Fig. 1). This enzyme is capable of hydrolyzing the various P–X bonds; P–O, P–S, P–F, P–CN, and P–N bonds *(4–6)*, and has been reported to be stereospecific for organophosphorous esters containing chiral phospho-

*Author to whom all correspondence and reprint requests should be addressed.

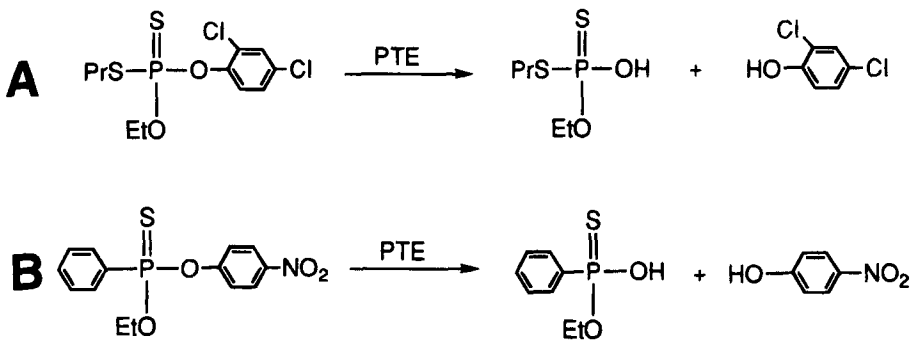

Fig. 1. The hydrolysis of organophosphorous esters, prothiofos **(A)** and EPN **(B)**, by PTE

rous atom *(7,8)* However, the enantiospecificity of PTE toward other chiral centers than chiral phosphorous atom has not yet been reported. The elucidation and application of such enzymatic characteristics will enable us to design a process for the preparation of chiral organophosphate insecticides by kinetic optical resolution of racemic compounds by PTE-catalyzed hydrolysis.

In this article, we investigate the site of chiral recognition by PTE using various type of organophosphorous esters containing chiral phosphorous and carbon atoms (Fig. 2).

MATERIALS AND METHODS

Chemicals

Prothiofos, phenthoate, and vamidothion were purchased from Nanogen Co. (Iza, UK), and ethyl 4-nitrophenyl phenylphosphonothioate (EPN) from Dr. Ehrenstorfer GmbH (Augusburg, Germany). Diethyl α-methylbenzyl phosphate and diethyl α-methylbenzyl phosphamide were prepared from chlorodiethyl phosphate, and the corresponding α-methylbenzyl alcohol and α-methylbenzylamine in the presence of triethylamine. These synthetic derivatives were purified with silica gel column chromatography. All analytical data are as follows: Diethyl α-methylbenzyl phosphate; ^{1}H-NMR (δ, $CDCl_3$) 1.22 (t, CH_3, 6H), 1.75 (d, CH_3, 3H), 3.95–4.05 (m, CH_2, 4H), 4.80–4.90 (m, CH, 1H), 7.10–7.15 (m, ArH, 10H) ppm, ^{31}P-NMR (δ, $CDCl_3$) –1.55 ppm. Diethyl α-methylbenzyl phosphamide, ^{1}H-NMR (δ, $CDCl_3$) 1.20 (t, CH_3, 6H), 1.80 (d, CH_3, 3H), 4.00–4.15 (m, CH_2, 4H), 5.10–5.20 (m, CH, 1H), 6.80–6.85 (m, NH, 1H), 7.10–7.15 (m, ArH, 10H) ppm, ^{31}P-NMR (δ, $CDCl_3$) –1.80 ppm. These racemic esters were analyzed by HPLC with chiral reversed-phase column (chiracel OJ-R, 4.6 × 150 mm, Daicel, Osaka, Japan). All enantiomers were separated in an isocratical condition (methanol:water, 93:7) at 0.7

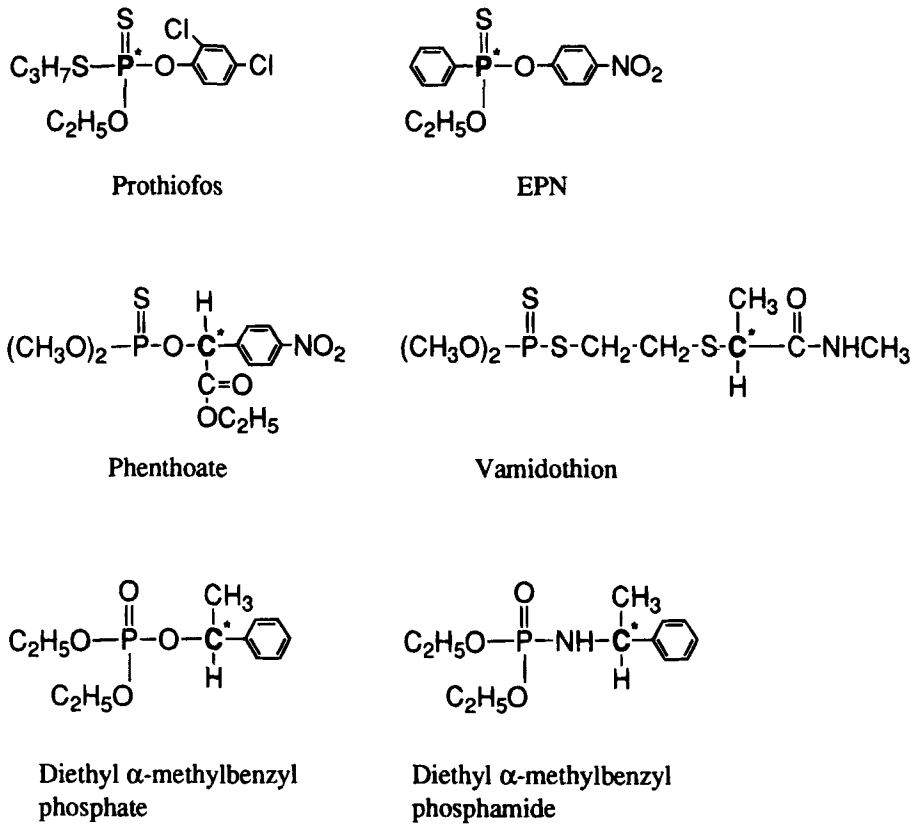

Fig. 2. Various organophosphorous esters containing chiral phosphorous and carbon atoms (asterisk) used in this study.

mL/min. Also, the resolved enantiomers of prothiofos and EPN were estimated from their optical rotations by polarimeter measurement (DIP-1000, Jasco, Tokyo, Japan). The earliest eluting peak in the chromatogram of prothifos by chiral HPLC was confirmed to be the (+)-enantiomer, and the later eluting peak was the (–)-enantiomer. Similarly, the earliest eluting EPN was confirmed to be the (+)-enantiomer, and the later eluting EPN was the (–)-enantiomer. In addition, the configuration of (+)-EPN is corresponded to the (*R*)-form, and (–)-EPN is the (*S*)-form, as previously reported *(7)*. The enantiomers of phenthoate, vamidothion, diethyl α-methylbenzyl phosphate, and diethyl α-methylbenzyl phosphamide were confirmed only by the separated two peaks on chromatography.

PTE Preparation

PTE was prepared from *Flavobacterium* sp. ATCC 27551, as follows. Cells were cultivated aerobically in a L-broth medium supplemented with 0.01 m*M* $ZnCl_2$, at 30°C for 21 h. Cells were then washed twice in 50 m*M*, pH 7.2 potassium phosphate buffer, and resuspended in 20% sucrose, 0.3*M*

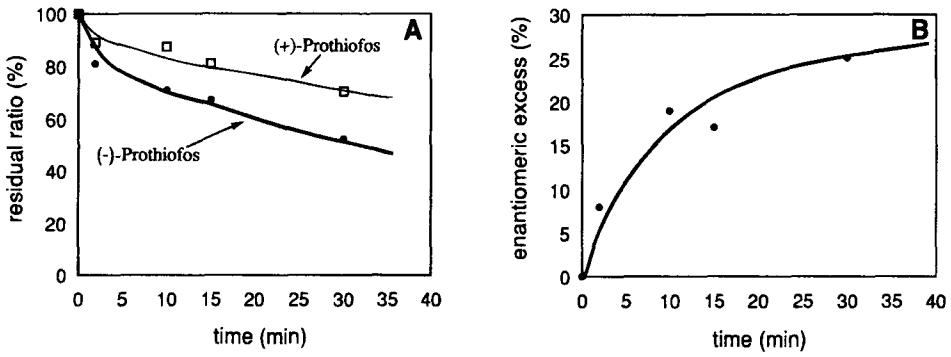

Fig. 3. The time-course of hydrolysis **(A)** and optical resolution **(B)** of prothiofos.

Tris-HCl (pH 8.1), 1 m*M* EDTA, and 0.5 m*M* $MgCl_2$. After centrifugation (5000*g* at 4°C), the cell pellet was decanted and resuspended in the residual liquid. From periplasmic space, PTE was extracted by osmotic shock by adding in ice-cold 0.5 m*M* $MgCl_2$. After incubation and centrifugation (10,000*g* at 4°C), the supernatant was dialyzed against 20 m*M*, pH 8.0, Tris-HCl buffer for 12 h at 4°C. This solution, containing 60 U/mL PTE activity, was then lyophilized and kept at –30°C until usage. PTE activity was determined by measuring the rate of liberation of 4-nitrophenol from tris-4-nitophenyl phosphate in the presence of PTE in 50 m*M* glycine-NaOH buffer (pH 9.5) at 25°C, by monitoring absorbance increase at 410 nm, by a spectrophotometer (UV-160A, Shimadzu, Japan).

Enzymatic Reaction of Racemic Organophosphorous Ester

Typical kinetic resolution of organophosphorous esters was carried out as follows. To a solution of 9.6 mg of prothiofos (10 m*M*) in 3 mL of 50 m*M* glycine-NaOH buffer (pH 9.5) was added 11 mg of lyophilized PTE corresponding to 30 U. The reaction was stirred at room temperature and was monitored by HPLC with a chiral reversed-phase column. The kinetic resolution of esters was estimated with the comparison of the earliest eluting peak and the later eluting peak on chiral HPLC.

RESULTS AND DISCUSSION

Figures 3 and 4 show kinetic resolution of racemic prothiofos and EPN by PTE. These esters have chiral phosphorous atoms that are attacked with nucleophile during hydrolysis. The prothiofos of (–)-enantiomer (later eluting) was degraded faster than (+)-enantiomer (earlier eluting) (Fig. 3A). The highest enantiomeric excess attained was 25% after 30 min (Fig. 3B). Similarly, for EPN (–)-enantiomer (later eluting) was degraded faster than (+)-enantiomer (earlier eluting) (Figs. 4A). The highest enantiomeric excess attained was 42% after 45 min (Fig. 4B). With regard to

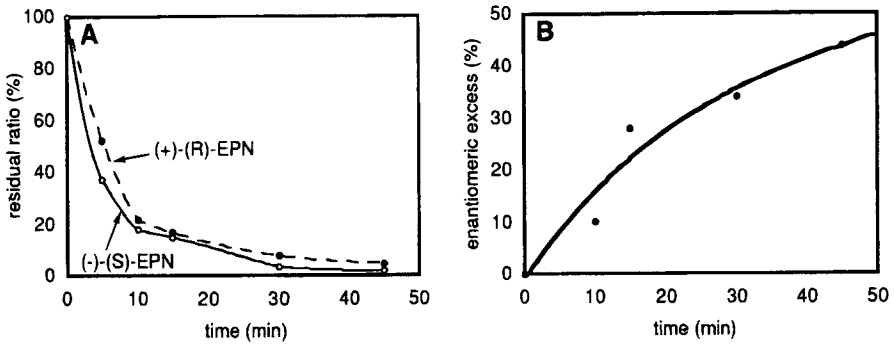

Fig. 4. The time-course of hydrolysis **(A)** and optical resolution **(B)** of EPN.

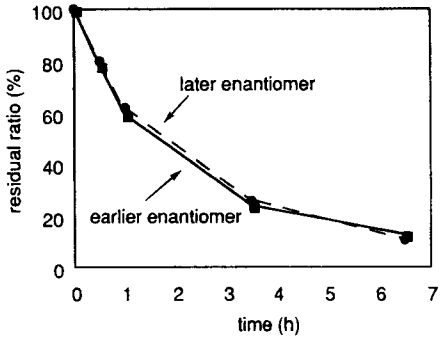

Fig. 5. The time-course of hydrolysis of phenthoate.

EPN, the predominant hydrolysis of (–)-EPN by PTE from *Pseudomonas diminuta* had been demonstrated by Lewis et al. *(7)*. EPN was degraded faster than prothiofos, indicating the difference in substrate specificity of prothiofos and EPN with PTE. These results show that the resolution with PTE has been performed stereospecifically.

Phenthoate, vamidothion, diethyl α-methylbenzyl phosphate, and diethyl α-methylbenzyl phosphamide were tested for PTE hydrolysis as organophosphorous esters having the chiral atom. The chiral center of these esters is located on the carbon atom closest to the phosphorous atom hydrolyzed. These results are shown in Figs. 5, and 6, and Table 1. Every substrate was hydrolyzed with PTE, but the stereospecificity was only found in diethyl α-methylbenzyl phosphamide hydrolysis. The earliest eluting enantiomer of diethyl α-methylbenzyl phosphamide was present in slight excess compared to the later eluting enantiomer (6% enantiomeric excess), whereas no stereospecificity was observed when phenthoate, vamidothion, and diethyl α-methylbenzyl phosphate were used as a substrate.

These results suggest that PTE mainly recognizes the chiral center on phosphorous atom. Recently, Vanhooke et al. reported about the stereo-

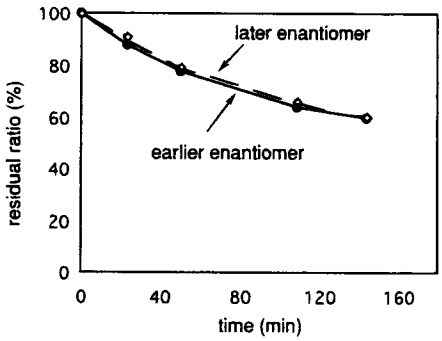

Fig. 6. The time-course of hydrolysis of vamidothion.

Table 1
Hydrolysis of Racemic Phosphorous Esters by Phosphotriesterase[a,b]

Substrate	Residual ratio (%)		e.e. (%)
$(C_2H_5O)_2-\overset{O}{\overset{\|}{P}}-NH-\overset{CH_3}{\overset{\|}{C}}H-$⬡	earliest enantiomer	73	6
	later enantiomer	65	
$(C_2H_5O)_2-\overset{O}{\overset{\|}{P}}-O-\overset{CH_3}{\overset{\|}{C}}H-$⬡	earliest enantiomer	70	0
	later enantiomer	70	

[a] The reaction was performed in glycine-NaOH buffer (pH 8.0) at 40°C at 24 h.
[b] The earliest and the later enantiomers were separated with chiral HPLC.

chemical mechanism of PTE hydrolysis reaction from the binding model of the inhibitor and the enzyme *(9)*. It was suggested that the phosphoryl group of the substrate was positioned within the PTE active site, but the leaving group as a 4-nitrophenyl group of prothiofos and EPN was located in the solvent rather than the interior of PTE. Therefore, phenthoate, vamidothion, and diethyl α-methylbenzyl phosphate containing the chiral center at the leaving group could not be recognized sufficiently by PTE. With diethyl α-methylbenzyl phosphamide, the small value of e.e. is supposed to be the diastereomeric effect by the double-bond character of phosphamide linkage.

CONCLUSION

We have shown that PTE chiral recognition is limited to organophosphorous esters having the chiral center on the phosphorous atoms, which is attacked with nucleophile, not having the chiral center on the other atoms of the leaving group. Therefore, PTE can be utilized for the synthesis of their chiral organophosphorous esters.

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