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Improvement of Enantioselectivity of Chiral Organophosphate Insecticide Hydrolysis by Bacterial Phosphotriesterase

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Abstract

The bacterial phosphotriesterase (PTE) isolated from *Flavobacterium* sp. can catalyze the cleavage of the P-O bond in a variety of organophosphate triesters and has been shown to be an effective catalyst for the degradation of toxic organophosphate esters. Ethyl 4-nitrophenyl phenylphosphonothioate (EPN) is a chiral organophosphate. Optical isomers of EPN show differences in their toxicity. R-EPN is known to be more toxic to hens and houseflies than S-EPN. We determined the K_i value of each enantiomer toward electric eel acetylcholinesterase. R-EPN (K_i = 6 μ*M*) inhibited acetylcholinesterase much more effectively than S-EPN (K_i = 52 μ*M*) did in vitro. Since PTE has been found to hydrolyze only the S-isomer of EPN, we attempted to alter the enantioselectivity of PTE in order to degrade toxic EPN enantiomer effectively. When PTE hydrolyzed EPN in the presence of dimethyl sulfoxide (DMSO), enzymatic activity toward S-EPN decreased linearly, but enzymatic activity toward R-EPN increased as a function of DMSO concentration. At 20% DMSO, the maximum activity was observed. The kinetic parameters of PTE to EPN isomers clearly indicated that in the presence of 20% DMSO, the enantioselectivity of PTE changed. The K_m value for R-EPN decreased from 0.24 to 0.03 m*M*, and the V_{max} value increased from 0.25 to 0.60 U/mg of protein. V_{max}/K_m values indicated that PTE preferred R-EPN over S-EPN in the presence of DMSO by a factor of 2.

Index Entries: Enantioselectivity; organophosphate insecticide; detoxification; phosphotriesterase.

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Introduction

The bacterial phosphotriesterase (PTE) is isolated from *Pseudomonas diminuta* and *Flavobacterium* sp. *(1,2)*. PTE is composed of two identical subunits with a molecular weight of 39,000 and is membrane associated *(3)*. Two divalent metal ions per monomer are essential for full catalytic activity. Because it is naturally isolated, PTE contains zinc ions *(4)*. PTE can catalyze the cleavage of the P-O bond in a variety of organophosphate triesters *(5)*. Therefore, it has been shown to be an effective catalyst for the degradation of toxic organophosphate esters *(6–9)*.

Ethyl 4-nitrophenyl phenylphosphonothioate (EPN) is a chiral organophosphate. Optical isomers of EPN show differences in their biological activities. There is no information on the in vitro toxicity of EPN isomers, such as K_i values for acetylcholinesterase (AChE). However, in vivo toxicity data, based on LD_{50}, show that R-EPN is more toxic to hens and houseflies than S-EPN *(10)*. PTE has been found to hydrolyze only the S-isomer of EPN *(1,11)* (Fig. 1). The R-EPN enantiomer is not hydrolyzed by the enzyme at any appreciable rate. These results suggested that the more toxic isomer would be left when PTE is employed for EPN detoxification.

It is known that a variety of enzymes are catalytically active in organic solvents and that some of them are utilized for the transformation of water-insoluble substrates and peptide synthesis. Previously, we investigated PTE catalyzed transesterification in organic solvent *(12)*. PTE maintained high catalytic activity in dimethyl sulfoxide (DMSO).

PTE would be an effective catalyst for the degradation of toxic chiral organophosphate esters if its catalytic activity toward R-isomer could be improved. In this study, we attempted to alter the enantioselectivity of PTE by using an organic solvent, DMSO. First, we investigated the K_i value of EPN enantiomers toward AChE to clarify the in vitro toxicity of each EPN enantiomer. We then showed that in the presence of DMSO, PTE catalyzed the hydrolysis of R-isomer of EPN effectively.

Materials and Methods

Chemicals

EPN and AChE from electric eel were purchased from Sigma (St. Louis, MO). 5,5'-Dithiobis-2-nitrobenzoic acid (DTNB), paraoxon, and acetylthiocholine were from Wako (Osaka, Japan). DMSO was from Kanto (Tokyo, Japan). All other reagents were of analytical grade.

AChE Inhibition by EPN

AChE activity was measured based on a DTNB method. AChE (188 μL) (400 U) dissolved in 100 m*M* phosphate buffer (pH 7.0) was mixed with 2 μL of various concentrations of EPN and incubated for 5 min. After adding 5 μL of 20 m*M* acetyl thiocholine and 5 μL of 18 m*M* DTNB, the increase

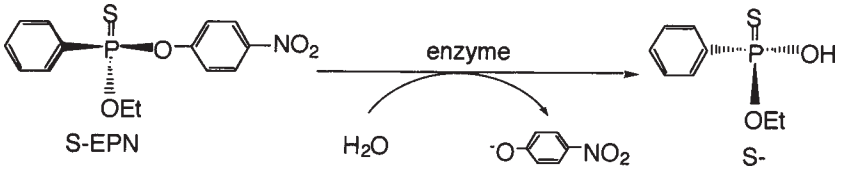

Fig. 1. Enantioselective hydrolysis of EPN with PTE 1.

Table 1
In Vitro and In Vivo Toxicity of EPN Enantiomers

	S-EPN	R-EPN	Racemic EPN
K_i (μ*M*) for AChE from electric eel	52	5.8	8.6
LD_{50} (mg/kg)[10] for hen, intraperitoneally	47	12.0	17.0

of absorbance at 410 nm was measured. The inhibition constant, K_i, for AChE was calculated based on Dixon plot.

Enzymatic Hydrolysis of EPN by PTE

Partially purified PTE of *Flavobacterium* sp. was prepared according to a previous report *(12)*. Two microliters of dioxane solution of EPN and 10 μL of 70 U/μL of PTE were mixed with 188 μL of 50 m*M* glycine/NaOH buffer (pH 9.5). The increase in absorbance at 410 nm owing to the release of *p*-nitrophenol by the hydrolysis of EPN was measured. One unit of enzyme was defined as the hydrolysis of 1 μmol of paraoxon/min under standard assay conditions.

Each EPN isomer concentration during enzymatic hydrolysis by PTE was measured as follows. Eight hundred and ninety microliters of 50 m*M* glycine/NaOH buffer (pH 9.5), 100 μL of PTE (70 U/μL), and 10 μL of racemic EPN were mixed and incubated. Every 10–30 min, 40 μL of the reaction mixture were sampled and analyzed by chiral reversed-phase high-performance liquid chromatography column (CHIRALCEL OJ-R, 4.6 mm ϕ × 150 mm, Daicel, Tokyo, Japan) with the eluent composed of methanol:water (9:1). The isomers were identified as shown in previous reports *(1,12)*.

Results

AChE Inhibition by EPN

Although R-EPN is known to be more toxic to hens and houseflies than the S-isomer in vivo *(10)*, there were no values in the literature K_i values for AChE. Therefore, we measured the inhibitory effect of EPN enantiomers on electric eel AChE activity to clarify the in vitro toxicity of EPN. The inhibition constant, K_i, was 6 μ*M* for R-EPN, 52 μ*M* for S-EPN, and 8.6 μ*M* for racemic EPN (Table 1). These K_i values indicated that the R-isomer

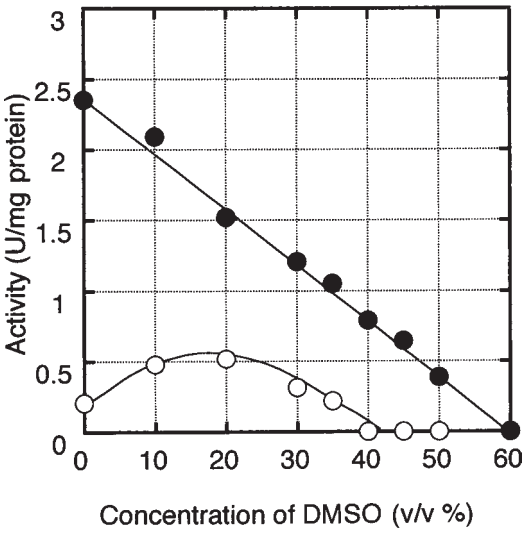

Fig. 2. Effect of DMSO concentration on PTE activity with R-EPN (○) or S-EPN (●) as the substrate. Enzyme activity was measured in a 50 m*M* glycine/NaOH buffer (pH 9.5) with 0–60% (v/v) DMSO.

of EPN inhibited AChE much more effectively than S-EPN did. R-EPN is nine times more toxic than S-EPN in vitro, and the toxicity of racemic EPN is mainly owing to R-EPN. Therefore, R-EPN must be degraded quickly for effective detoxification of EPN.

Effect of DMSO on Enzymatic Hydrolysis of EPN

Figure 2 shows the effect of DMSO on PTE activity toward 1 m*M* of each EPN enantiomer. In the absence of DMSO, enzymatic activity toward S-EPN is about 10 times higher than that toward R-EPN. As DMSO concentration increased, enzymatic activity toward S-EPN decreased linearly. However, enzymatic activity toward R-EPN was increased, and at 20% DMSO, the maximum activity was observed, which was 2.5-fold that of initial activity. This result indicated that DMSO has the ability to alter the enantioselectivity of PTE and effectively increases the rate of degradation of the more toxic R-isomer of EPN.

Enzymatic Hydrolysis of Racemic EPN by PTE

Figure 3A,B shows the time course of hydrolysis of racemic EPN using PTE. S-EPN was completely degraded but most of the R-EPN remained. Racemic EPN concentration was decreased to 50% in the first 10 min but was not decreased further on longer incubation. These results indicated that PTE has limited enzymatic activity against a racemic mixture of EPN, most likely because of selectivity toward the S-isomer. This is clearly shown by the results in Fig. 3 because the S-isomer is fully degraded whereas the PTE enzyme has almost no activity toward the R-isomer.

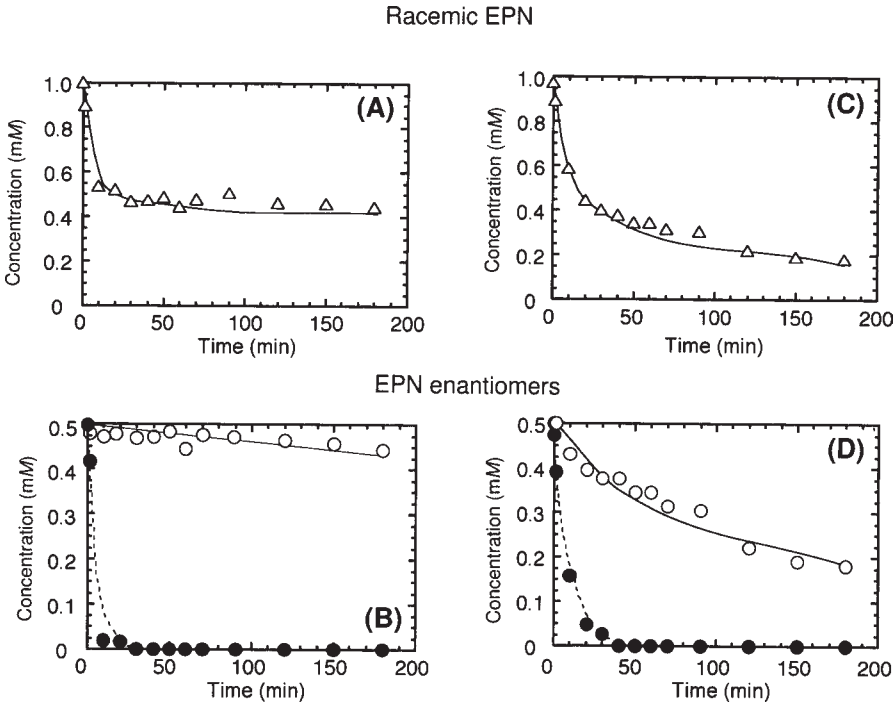

Fig. 3. Time course of racemic EPN hydrolysis by PTE. **(A)** Racemic EPN concentration during hydrolysis of racemic EPN by PTE in the absence of DMSO; **(B)** R-EPN (○) and S-EPN (●) concentration during hydrolysis of racemic EPN by PTE in the absence of DMSO; **(C)** racemic EPN concentration during hydrolysis of racemic EPN by PTE in the presence of 20% DMSO; **(D)** R-EPN (○) and S-EPN (●) concentration during hydrolysis of racemic EPN by PTE in the presence of 20% DMSO.

Figure 3C,D shows the time course of hydrolysis of racemic EPN using PTE in the presence of 20% DMSO. S-EPN was completely degraded within 40 min, and 60% of R-EPN was degraded in 180 min.

Discussion

Why did DMSO alter the enantioselectivity of PTE? Fitzpatrick and Klibanov *(13)* reported that enantioselectivity of the protease subtilisin Carlsberg in the transesterification was greatly affected by the solvent. In the absence of organic solvent, subtilisin Carlsberg preferred R-isomer, but the addition of organic solvent altered the stereospecificity. The mechanism was postulated that the protein's flexibility increases (because of a higher dielectric constant of the solvent) and the steric constraints become more forgiving, thereby allowing for a greater reactivity of the R-enantiomer. Griebenow and Klibanov *(14)* reported that DMSO destroyed the secondary structure of subtilisin Carlsberg, lowering the contents of α-helices and β-sheets *(14)*. In our case, DMSO could increase the enzyme's flexibility, and the reactivity toward R-isomer increased. On the other hand,

Table 2
Kinetic Constants of PTE

	K_m (m*M*)	V_{max} (U/mg protein)	V_{max}/K_m
In the absence of DMSO[a]			
S-EPN	0.033	2.30	70
R-EPN	0.24	0.25	1
Racemic EPN	0.10	1.25	12
In the presence of DMSO[b]			
S-EPN	0.18	1.80	10
R-EPN	0.03	0.60	20
Racemic EPN	0.10	1.11	10

[a]Enzyme activity was measured in a 50 m*M* glycine/NaOH buffer (pH 9.5).
[b]Enzyme activity was measured in a 50 m*M* glycine/NaOH buffer (pH 9.5), 20% (v/v) DMSO.

there remains the possibility that the enzyme activity itself was not affected by DMSO but that the structure or stability of the substrate, EPN, was changed by DMSO.

Table 2 presents the kinetic parameters of PTE toward EPN isomers in both the absence and presence of DMSO. In the absence of DMSO, the V_{max}/K_m value of PTE to EPN isomers clearly indicated that PTE preferred S-EPN 70-fold more than R-EPN. Kinetic parameters of PTE to each EPN isomer in the presence of 20% DMSO were different from those measured in the absence of DMSO. Kinetic parameters of PTE to each EPN isomer clearly indicated that in the presence of 20% DMSO, the enantioselectivity of PTE changed. In the presence of 20% DMSO, K_m for S-EPN increased sixfold and V_{max} for S-EPN decreased to about 80% of V_{max} in the absence of DMSO. K_m for R-EPN decreased to about 15% of the value in the absence of DMSO, and V_{max} for R-EPN increased 2.4-fold. Judging from the V_{max}/K_m values, PTE preferred R-EPN by a factor of two over S-EPN in the presence of 20% DMSO. Considering that in the absence of DMSO PTE preferred S-EPN 70-fold more than R-EPN, catalytic activity toward R-EPN was greatly enhanced by the addition of DMSO.

Our results from analyzing the degradation of racemic EPN by PTE allowed us to estimate the decrease in toxicity of EPN toward AChE as a measure of how the PTE enzyme can detoxify these organophosphates. In the absence of DMSO, the half-life for inhibiting AChE to 50% of the initial value required 760 min. In the presence of 20% DMSO, achieving the 50% level of initial inhibition occurred in 90 min. This suggests that the addition of DMSO shortened the detoxification period eightfold.

Conclusion

Since PTE cannot degrade R-EPN effectively, we tried to alter the enantioselectivity of PTE. We determined AChE inhibition constants of EPN enantiomers and clarified that R-EPN is a more toxic enantiomer.

We succeeded at showing that in the presence of 20% DMSO, R-EPN was effectively degraded by PTE and that the estimated EPN detoxification period by PTE was shortened.

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