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Improving the Thermostability of a Methyl Parathion Hydrolase by Adding the Ionic Bond on Protein Surface

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Abstract The thermostability of the methyl parathion hydrolase (MPH_OCH) from *Ochrobactrum* sp. M231 was improved using site-directed mutagenesis. Two prolines (Pro76 and Pro78) located on the protein surface were selected for mutations after inspection of the sequence alignment of MPH_OCH and OPHC2, a thermostable organophosphorus hydrolase from *Pseudomonas pseudoalcaligenes* C2-1. The temperature of the double-point mutant (P76D/P78K) at which the mutant lost 50% of its activity (T50) was approximately 68 °C, which is higher than that of WT enzyme (64 °C), P76D (67 °C), and P78K (59 °C). Structural analysis of P76D/P78K indicated that the substituted residues (Asp76 and Lys78) could generate an ionic bond and increase the structural electrostatic energy, which could then increase the stability of the protein. These results also suggest that the thermal stability of proteins could be improved by adding the ionic bond on protein surface.

Keywords Methyl parathion hydrolase · Site-directed mutagenesis · Thermostability · Ionic bond

Introduction

Improving the thermostability of enzymes used commercially or industrially, would expand their applicability and they have greater industrial potential owing to increased enzymatic

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efficiency and cost-effectiveness [13]. However, until now, there are no specific rules or methods to improve protein thermostability [11, 17]. Factors that influence the thermostability of a protein have been determined by comparing mesozyme and thermozyme sequences and/or structures [8, 10]. These factors include increasing intramolecular interactions, optimizing electrostatic charges, making the structure more compact, increasing conformational rigidity, and improving the stability of α-helices [15, 16, 18].

The thermostability of a protein may also be improved by increasing the number and/or the strength of ion pairs [12] or by optimizing the surface electrostatic distribution [7]. Moreover, this method usually does not affect the function of the modified protein and thus, might be widely used to improve protein thermostability [7].

Methyl parathion hydrolase (MPH; EC 3.1.8.1) is a class of enzyme that could efficiently degrade the methyl parathion, an organophosphate pesticide that has been used extensively in agriculture [5, 6, 16, 21–23]. In this study, we focused on a MPH from *Ochrobactrum* sp. M231, designated as methyl parathion hydrolase (MPH_OCH; Genbank No: ACC63894). The enzyme could degrade methyl parathion with great efficiency but had poor thermostability, and was thus selected to see if its thermostability could be improved [20]. However, another methyl parathion hydrolase, OPHC2 (Genbank No: CAE53631) from *Pseudomonas pseudoalcaligenes* C2-1 [3], has a greater thermostability than MPH_OCH. The sequence identity and similarity between OPHC2 and MPH_OCH are 48% and 66%, respectively. After incubation at 60 °C for 30 min, 60% of the MPH_OCH activity is lost [20]. However, OPHC2 retains almost 80% of its enzymatic activity under the same conditions [3]. At 70 °C, the enzymatic activity of MPH_OCH rapidly decreases; whereas, OPHC2 retains 50% of its activity after a 30-min incubation.

In this study, the sequences of MPH_OCH and OPHC2 were aligned and two residues at positions 76 and 78 located at the surface, which were found in a misaligned region, were substituted, either individually or at the same time, with the charged amino acids Asp and Lys, respectively. As a result, the double-point mutant (P76D/P78K) was found to be more thermostable than was wild-type MPH_OCH.

Materials and Methods

Strains, Plasmids, and Chemicals

Escherichia coli JM109 (Promega, Madison, WI, USA) and BL21 (DE3) (Novagen, Darmstadt, Germany) were used for recombinant plasmid amplification and protein expression, respectively. The plasmids of peasy-T3 (Transgen, Beijing, China) and pET-30a(+) (Novagen, Darmstadt, Germany) were used for cloning and expression, respectively. The *Ochrobactrum* sp. 231 strain was isolated from pesticide factory soil (Tianjin, China) and stored in our lab. All restriction enzymes were obtained from TaKaRa (Otsu, Japan). Isopropyl-β-D-thiogalactopyranoside, kanamycin, and imidazole were purchased from Ameresco (Tully, NY, USA). All chemicals were of analytical grade.

Cloning, Expression, Purification, and Quantification of Recombinant MPH_OCH

The gene for MPH_OCH was amplified using the MPH_PF and MPH_PR primers (Supplementary Table 1). The polymerase chain reaction (PCR) product was gel purified, digested with *Eco*RI and *Not*I, and cloned into the corresponding sites of pET-30a(+). The

recombinant plasmid pET-MPH was then transformed into *E. coli* BL21 (DE3)-competent cells. A positive colony harboring *mph* gene was confirmed by DNA sequencing (ABI-3730X, USA) in the State Key Laboratory of Crop Genetic Improvement, Chinese Academy of Agricultural Sciences. *E. coli* harboring pET-MPH were incubated overnight in LB liquid medium at 37 °C and then transferred to fresh LB medium (1:100 dilution) for incubation at 37 °C until the culture had an A_{600} of ~0.6. After induction with isopropyl-β-D-1-thiogalactopyranoside (final concentration, 1 mM), the culture was incubated at 16 °C for an additional 12 h. Then, the cells were collected by centrifugation, disrupted by sonication, and recombinant MPH was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [14]. The recombinant $(His)_6$-tagged MPH was purified by a Ni-NTA His-bind™ Resin column (Novagen) according to the manufacturer's instructions. The final concentration of the purified protein was determined using the reagents of a Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA, USA).

Site-Directed Mutagenesis

Site-directed mutagenesis was performed using overlapping PCR mutagenesis [14]. The primers for the mutagenesis are listed in Supplementary Table 1. The expected mutations were confirmed by DNA sequencing in the State Key Laboratory of Crop Genetic Improvement, Chinese Academy of Agricultural Sciences. The methods for expression and purification of the mutants were the same as those described for wild-type enzyme.

Standard Enzyme Assay and Determination of Kinetic Parameters

The methods for the standard enzyme assay and determination of kinetic parameters were the same as to the reference [16].

Thermostability assay of WT and mutant enzymes.

All of the purified enzymes were diluted to 120 μg/mL with 50 mM Tris buffer (pH 8.0). The diluted enzymes were incubated at various temperatures ranging from 50 °C to 70 °C for 10 min. In addition, the diluted enzymes were also incubated at 64 °C and 70 °C for different time periods. Immediately after heating, the enzymes were placed on ice for 30 min. The residual MPH activity was measured using the assay described above with at least three samples run in parallel.

Homology Modeling of MPH_OCH and its Mutants

The models of WT and mutant enzymes were constructed and optimized by the Discovery Studio 2.5.5 software (Accelrys, USA), which was the same as to the reference [16].

Structure Energy Calculations

The structures of the WT and mutant enzymes were minimized by the Discovery Studio 2.5.5 software (Accelrys Software Inc., San Diego, CA, USA) with the "Minimization protocol." The minimization algorithms of the steepest descent and conjugate gradient methods were used with a generalized born implicit solvent model [2]. The run steps of each minimization were set at 5,000 steps. Then the potential energy, van der Waals energy, and electrostatic energy for the structures of the WT and mutant enzymes were determined with the Discovery Studio 2.5.5 software using the "calculate energy protocol."

Results and Discussion

Selection of the Mutation Sites in MPH_OCH

The amino acid sequences for MPH_OCH and OPHC2 were compared using ClustalW (Fig. 1) and their sequence identity and similarity were found to be 48% and 66%, respectively. As shown in Fig. 1, the two proteins share a similar sequence with the exception that residues 71–80 differ. In this region, OPHC2 contains more charged residues than does MPH_OCH.

The 3-D model of MPH_OCH was constructed using Discovery Studio 2.5.5 software and, as the template, the crystal structure of MPH_WBC3, which can be described as a αβ/βα sandwich typical of the metallo-hydrolase/oxidoreductase fold [6]. The final model of MPH_OCH possessed good stereochemical quality as determined by Discovery Studio 2.5.5, which has only one residue (Asp112) with the backbone Φ and Ψ dihedral angles located out of the generously allowed regions of the Ramachandran plot. As shown in Fig. 2, the region is distant from the active center of MPH_OCH. Therefore, mutations in this region were not expected to affect the function of the enzyme. As surface residues can affect protein thermostability [7], residues located between residues 71 and 80 were selected as potential mutation sites. In this study, we focused on two prolines (Pro76 and Pro78), which were corresponding to a pair of charged residues (Asp76 and Lys78) in OPHC2, locating on the structure surface of MPH_OCH as the mutation targets. Therefore, we constructed four mutants including MPHR10 (replacing "KRLNQPAPKT" of MPH_OCH to "LLKGIDDKDL" of OPHC2 at residues from 71–80), MPHP76D (Pro substitution at residue 76), MPHP78K (Pro substitution at residue 78), and MPHP76D_P78K (P76D, P78K double-point mutant).

Purification and Enzymatic Activity of Wild-Type and Mutant MPHs

The genes encoding the wild-type and mutant MPHs were cloned, expressed, and purified using Ni-NTA affinity chromatography. As a result, the mutant MPHR10 had weak enzyme activity and was not determined the kinetic characterization and thermostability of the mutant. The purified proteins migrated as single bands upon SDS-PAGE and the molecular mass was estimated as 33 kDa (data not shown).

Kinetic Characterization of WT and Mutant Enzymes

The kinetic parameters of WT and the three mutant enzymes were measured as described in the "Materials and Methods" section, and the results are shown in Table 1. All of the

Fig. 1 Sequence alignment of MPH_OCH and OPHC2 showing the notable sequence differences between residues 71 and 80

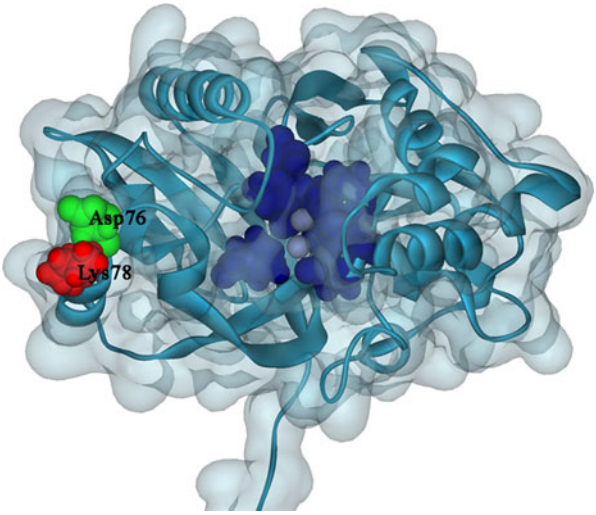

Fig. 2 The 3-D model of MPHP76D_P78K. The mutated residues Asp76 and Lys78 are located on the protein surface. The key residues for the catalysis reaction and the two zinc ions were shown as the *blue* and *silver*

mutants have the activity of methyl parathion hydrolase. The mutant MPHP76D_P78K and MPHP76D have the similar overall catalytic efficiency (k_{cat}/K_m), which was higher than the WT enzyme, and the overall catalytic efficiency (k_{cat}/K_m) of mutant MPHP78K was lower than that of the WT enzyme.

Thermostability of the Wild-Type and Mutant MPHs

The thermostabilities of the wild-type and mutant MPHs were determined by measuring their residual activities after incubation for 10 min at various temperatures (Fig. 3). The temperature at which the mutant MPHP76D_P78K lost 50% of its activity (T_{50}) was approximately 68 °C, which is higher than that of WT enzyme (64 °C), MPHP76D (67 °C), and MPHP78K(59 °C). But the T_{50} of the mutant MPHP78K was lower than that of the WT enzyme.

To further investigate the thermostability of the WT and mutant enzymes, the enzymes were incubated at 64 °C and 70 °C for different periods and the residual activities of the enzymes were measured (Fig. 4). After incubation for 10 min at 64 °C, wild-type MPH, MPHP76D and MPHP78K retained 49%, 72%, and 25% activities, respectively.

Table 1 Comparison of properties of the wild-type and mutant enzymes

Enzyme	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} min^{-1})
MPH_OCH	252.8±12.6	76.3±4.1	3.3±0.3
MPHP76D_P78K	800.2±50.1	74.7±3.9	10.7±1.2
MPHP76D	689.8±39.0	68.2±3.6	10.1±1.1
MPHP78K	72.2±4.9	112.4±4.8	0.6±0.1

K_m and k_{cat} were calculated by nonlinear regression analysis using GraphPad Prism. All values are expressed as (mean±SD), based on three separate experiments

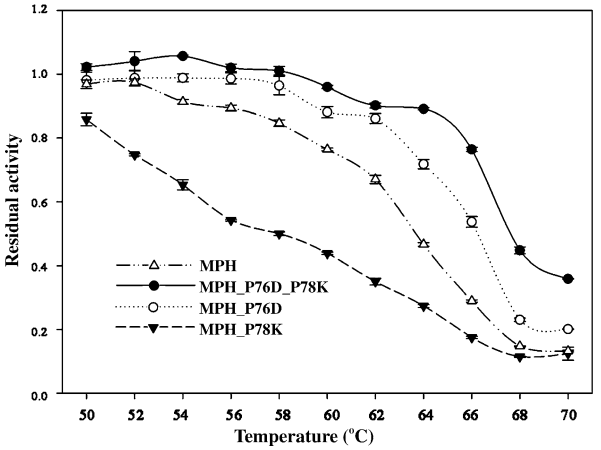

Fig. 3 Thermostability of wild-type and mutant enzymes (MPHP76K_P78K, MPHP76D, and MPHP78K). The thermal stability of the enzymes was determined by monitoring residual enzymatic activity after incubation for 10 min at various temperatures. Enzymatic activity was then assayed using the standard enzyme assay. Data points correspond to the mean values of three independent experiments

Conversely, MPHP76D_P78K retained 90% of its initial activity under the same temperature condition. After incubation at 70 °C for 10 min, wild-type MPH, MPHP76D_P78K, MPHP76D and MPHP78K retained 14%, 39%, 20%, and 11% activities, respectively. These results also suggested that MPHP76D_P78K is more thermostable than wild-type MPH.

Here, we have compared the sequence of MPH_OCH with other MPHs isolated from the *Plesiomonas* sp. M6 (Genbank No: AF338729) [5], *Pseudomonas* sp. WBC-3 (Genbank No: AAP06948) [9, 19] and *Stenotrophomonas* sp. YC-1 (Genbank No: DQ677027) [21]. As a result, the residues (residue numbers 76 and 78) of the other MPHs were the same as MPH_OCH, which were prolines. The result implied that the thermostability of other MPHs might be improved by designing the ionic bond on the protein surface. In a previous study, we have identified a disulfide bond in OPHC2, which had contribution on the thermostability of the OPHC2 [4]. In this study, we found that the ionic bond also has the contribution on the protein thermostability. After designing the ionic bond into the surface of MPH_OCH, the thermostability of MPH_OCH was improved. The results indicated that the proteins might employ multiple ways to maintain the thermostability.

Structure Analysis of the Wild-Type and Mutant MPHs

By replacing the prolines with the two charged residues, the thermostability of the enzyme was improved. The 3-D models of the MPH_OCH and MPHP76D_P78K were constructed using Discovery Studio 2.5.5 software with the standard modeling method. As shown in Fig. 2, the mutated residues are located on the surface of the protein and an ion pair between them can be formed. Moreover, as the 79th residue of MPH_OCH was a lysine, the generated residue D76 from the mutation P76D could have the electronic interaction with K79. As a result, the thermostability of mutant MPHP76D was also better than WT enzyme.

The structure energies of WT and mutant enzymes were also calculated with the CHARMm force field [1] by the software Discovery Studio 2.5.5 (Accelrys Software Inc., San Diego, CA, USA). As shown in Table 2, the potential energies of mutant MPHP76D_P78K and MPHP76D were 232.1 kcal/mol and 105.1 kcal/mol, which lower

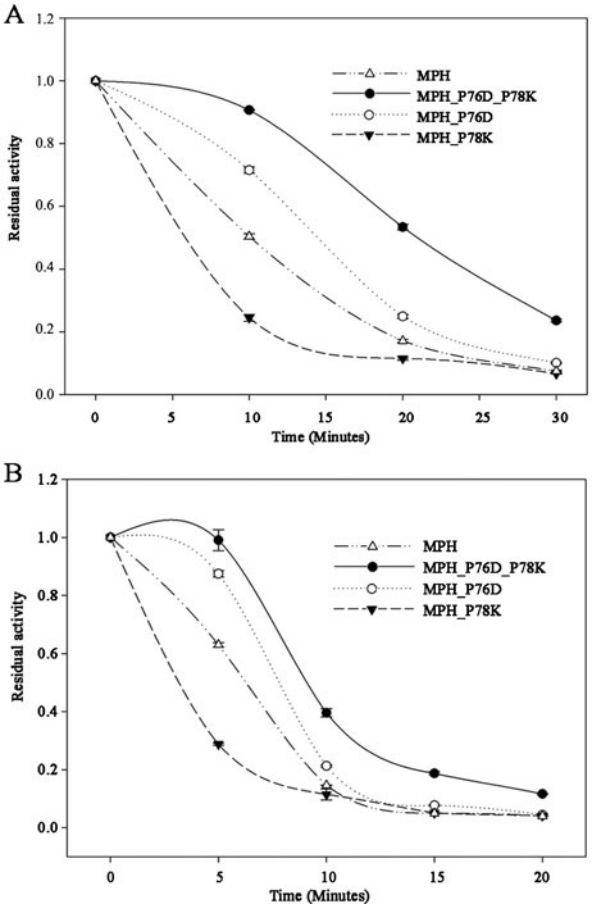

Fig. 4 Thermostability of MPH_OCH and the three mutants (MPHP76K_P78K, MPHP76D and MPHP78K) at **a** 64 °C and **b** 70 °C. Proteins were incubated at the indicated temperatures (between 10 and 30 min) and then tested for residual enzymatic activity. Data points correspond to the mean values of three independent experiments

Table 2 The potential energy, Van der Waals energy and electrostatic energy of the wild-type and mutant enzymes

	Potential energy		Van der Waals energy		Electrostatic energy	
	Value[a]	Difference[b]	Value	Difference	Value	Difference
MPH_OCH	−15,968.8	0.0	−2,321.9	0.0	−9,410.1	0.0
MPHP76D_P78K	−16,200.9	−232.1	−2,327.5	−5.6	−9,591.0	−180.9
MPHP76D	−16,073.9	−105.1	−2,319.6	2.3	−9,651.0	−240.9
MPHP78K	−15,864.4	104.4	−2,276.1	45.8	−9,442.6	−32.5

Calculation is based on the force field CHARMm. Values are in kcal/mol

[a] The corresponding energy value

[b] The energy difference between the protein and the WT MPH

than that of WT enzyme. It indicated that the structures of MPHP76D_P78K and MPHP76D were more stable than that of WT enzyme. The structure stabilities of MPHP76D_P78K and MPHP76D were mainly due to the enhanced electrostatic interaction, as the electrostatic energies of the MPHP76D_P78K and MPHP76D were lower than that of WT enzyme (Table 2). Although the electrostatic energy of the mutant MPHP78K was also lower than that of WT enzyme, the Van der Waals energy and the potential energy of the mutant MPHP78K were greater than those of WT enzyme. Therefore, the thermostability of the mutant MPHP78K was lower than that of WT enzyme.

Conclusion

The thermostability of MPH_OCH was improved by adding an ionic bond to the protein surface. Compared with mutations in a protein core, those that occur on the structure surface are less likely to disrupt the tertiary structure [7]. Therefore, an ion pair on the surface could increase the number of electrostatic interactions, which might improve the thermostability of MPH_OCH. This research also indicated that the thermostability of a protein could be enhanced by changing surface nonpolar residues to charged ones and by adding the ionic bond to the surface of a protein.

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