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Improved efficiency of a novel methyl parathion hydrolase using consensus approach

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ABSTRACT

A methyl parathion hydrolase (MPH) gene, *bjmpd*, was cloned from a newly isolated MP-degrading bacterial strain, *Burkholderia jiangsuensis* MP-1^T and heterologously expressed in *Escherichia coli* BL21 (DE3). Although the amino acid sequence of the *bjmpd*-encoded enzyme, named *Bj*MPH, differed from that of MPH from *Pseudomonas* sp. WBC-3 (*Ps*MPH) in only three residues, Ser132, Val247 and Ala267, a significantly higher specific activity towards MP was exhibited by *Bj*MPH than *Ps*MPH. Among them, Ala267 was identified as a key site affecting the catalytic efficiency, and it was rather conservative (Ala or Ser) in homologous proteins, suggesting that a simple substitution of the residue in conservative site with another conservative residue based on the consensus sequence approach might possibly enhance the catalytic efficiency of the MP-degrading enzyme. Inspired by such an observation, we identified a new mutant, *Bj*MPH$_{T64N}$, exhibiting 3.78-fold higher catalytic efficiency (k_{cat}/K_M) towards MP than its wild-type, reaching 4.20×10^6 M^{-1} s^{-1}. The mutant *Bj*MPH$_{T64N}$ also displayed enhanced reactivities (k_{cat}/K_M) towards other organophosphorus pesticides. Homology-modelling analysis indicates that enhanced polar contacts of the 64th residue in this mutant may contribute to stabilizing the structure of the enzyme and promote the interactions between enzyme and substrate. This study generated an efficient MP-degrading enzyme, and provides useful information for enhancing the catalytic efficiency of MPHs via conservative residue substitution based on the consensus approach.

1. Introduction

A great diversity of organophosphorus pesticides (OPs) are applied extensively and globally for crop protection in agriculture [1]. In general, the phosphorus is linked by a double bond to either an oxygen (P═O) in oxon OPs or a sulfur (P═S) in thion OPs [2]. Methyl parathion (*O*,*O*-dimethyl *O*-*p*-nitrophenol phosphorothioate, MP) is an extremely toxic organophosphate insecticide that is released into the environment during its manufacture and use [3]. Residual MP may result in severe environmental contamination involving water, air and soil [4]. The acute toxic effects of MP are related to inhibiting acetylcholinesterase in mammals including humans and insects [5], leading to serious health problems or even death [6]. The current physical and chemical approaches for eliminating pesticide residue have their own limitations, for instance energy consumption or secondary pollution [7,8].

In consideration of effectiveness and economy, enzymatic decontamination of MPs currently plays a key role. Increasing studies have focused on biodegradation using OP-degrading enzymes [9]. Two excellent categories of enzymes have been described frequently. Organophosphorus hydrolases (OPHs, E.C.3.1.8) encoded by the *opd* gene are distributed extensively in a variety of bacteria including *Flavobacterium* sp. strain ATCC27551 [10], *Pseudomonas diminuta* MG [11] and *Acinetobacter* sp. [12]. OPH enzymes have broad temperature and pH optima, and the capacity to degrade a wide range of OP compounds with similar structures to parathion [13]. Methyl parathion hydrolase (MPH, E.C.3.1. 8.1) encoded by the *mpd* gene (named for 'methyl parathion degradation') was first discovered and cloned from *Plesiomonas* sp. M6. Intriguingly, it showed no homology to the known *opd* genes [14]. Subsequently, *mpd* genes were isolated from various microorganisms, including *Pseudomonas* sp. strain WBC-3 [13], *Bacillus subtilis* WB800 [5], and *Sphingomonas* sp. strain Dsp-2 [15].

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It has been revealed that MPH degrades a broad spectrum of OPs [16,17], including methyl parathion [18], parathion and fenitrothion [15]. To understand the mechanism of the enzymatic hydrolysis, the crystal structure of MPH from *Pseudomonas* sp. WBC-3 (*Ps*MPH) was determined. MPH belongs to the metallo-β-lactamase family with an αββα fold and has conserved metal-coordinating residues in the binuclear (Zn^{2+} and Cd^{2+}) catalytic center. It was proposed that the amino acid residues located at the entrance to the catalytic center coordinate the two metal ions and form the aromatic cluster [19].

*Ps*MPH showed a relatively high catalytic efficiency for MP hydrolysis, with a k_{cat}/K_M of $9.92 \times 10^5\ M^{-1}\ s^{-1}$. However, it exhibited low catalytic activity towards other OPs [20]. To enhance the enzymatic activity of *Ps*MPH towards *p*-nitrophenyl diphenylphosphate, directed evolution was applied to obtain a triple mutant (Q81L/F196A/L273A) with 100-fold higher catalytic efficiency (k_{cat}/K_M) than the wild-type enzyme [18]. Recently, site saturation mutagenesis and DNA shuffling were performed to alter the substrate specificity of *Ps*MPH. One mutant exhibited a 100-fold increase in the k_{cat}/K_M value towards ethyl paraoxon ($6.00 \times 10^5\ M^{-1}\ s^{-1}$), with a decreased activity towards MP. During this evolution, a mutant was identified (L258I), which manifested a 2.5-fold increased k_{cat}/K_M value towards MP [17].

In our previous work, a novel and highly effective MP-degrading strain, *Burkholderia jiangsuensis* sp. nov. strain MP-1^T, was isolated [21]. However, the gene encoding the MP-degrading enzyme remained unknown. In this study, the *bjmpd* gene from *B. jiangsuensis* MP-1^T was cloned. Heterologous expression, purification, and characterization of recombinant *Bj*MPH were undertaken and kinetic parameters for MP hydrolysis were determined. Because of the higher specific activity of *Bj*MPH than its triple mutant *Bj*MPH$_{S132N/V247A/A267S}$, as a mimic of *Ps*MPH, key amino acid substitutions in conservative sites based on consensus sequence analysis was thought to be possible to evolve the MPH towards higher activity. To further improve the catalytic efficiency of *Bj*MPH, we introduced potentially beneficial mutations suggested by multiple sequence alignment and the best variant *Bj*MPH$_{T64N}$ with remarkably enhanced catalytic efficiency ($k_{cat}/K_M = 4.20 \times 10^6\ M^{-1}\ s^{-1}$) was obtained. The possible mechanism for the improved catalytic efficiency was also speculated based on homology modeling and molecular docking analyses.

2. Materials and methods

2.1. Chemicals and materials

All OPs (>99% purity) used in this study were purchased from Shanghai Pesticide Research Center. Methanol and acetonitrile were purchased from Tedia (Fairfield, OH, USA). All compounds were prepared in acetonitrile. All other reagents used in this work were of analytical grade. Restriction endonucleases and T4 DNA ligase were purchased from TaKaRa (Dalian, China). Culture conditions were prepared according to Liu et al. [21].

2.2. Cloning, expression and purification

The *bjmpd* gene of strain MP-1^T was amplified using a set of primers as previously described [22]. PCR products were double digested with restriction enzymes *Nde*I and *Xho*I and ligated into pET28a. The resultant plasmids were transformed into *Escherichia coli* BL21 (DE3) for expression. BL21 cells incubated overnight were subinoculated into 50 ml LB medium (10.0 g L^{-1} peptone, 10.0 g L^{-1} yeast extract, 5.0 g L^{-1} NaCl) containing 50 mg L^{-1} kanamycin and incubated aerobically at 37 °C for 16–18 h. When the optical density (OD_{600}) of culture broth reached 0.6–0.8, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.2 mM. The cells were incubated at 25 °C for a further 16 h, harvested by centrifugation at 7690 × *g* for 10 min at 4 °C, washed with saline, and resuspended in Tris-HCl buffer (50 mM, pH 9.0). The cell suspension was then disrupted by sonicating 99 times for 4 s periods at 6 s intervals. The resultant recombinant *Bj*MPH was purified by Ni-chelating affinity chromatography [23]. The expressed protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12% gel). Protein concentrations were quantified using the Bradford method [24].

2.3. Activity assays and kinetic parameters

A standard activity assay for MP degradation was performed in Tris-HCl buffer (50 mM, pH 9.0) at 30 °C. The hydrolysis of 0.5 mM MP was measured at 405 nm (ε_{405} = 17,700 $M^{-1}\ cm^{-1}$) [23]. The activities towards the other OPs were also determined spectrophotometrically at specific wavelengths [12,23]. All assays were performed in triplicate. Specific activities were defined as units (micromoles of substrate hydrolyzed per minute) per milligram of protein. Kinetic constants (K_M and k_{cat}) were acquired by measuring activity with substrate varying in concentration from 0.1 × K_M to 7 × K_M and fitting data to the Michaelis-Menten equation.

2.4. Thermostability and pH stability

The thermostability of the enzymes was determined by measuring the melting temperature (T_m) via circular dichroism (CD) spectroscopy. The purified protein samples (200 μg mL^{-1} each) dissolved in 50 mM potassium phosphate buffer (pH 8.0) were loaded into a 0.1 cm path-length cuvette, and the CD spectra were detected from 180 nm to 260 nm under varied temperatures (30–90 °C). The pH stabilities at pH 6.0 and pH 10.0 were respectively investigated by monitoring the residual activities of the purified protein incubated in two different buffers, citrate buffer (100 mM, pH 6.0) and glycine-NaOH buffer (100 mM, pH 10.0).

2.5. Multiple sequence alignment and site-directed mutagenesis

Sequence similarities of homologous proteins were determined using Uniprot (http://www.uniprot.org/). The multiple sequence alignment used ClustalW2 and ESPript 3.0 [25]. A phylogenetic tree was constructed using the neighbor-joining method [26] in MEGA version 6.0 [27]. The topology of the phylogenetic tree was evaluated using bootstrap values based on 1000 replications. Site-directed mutations of *Bj*MPH were performed using a QuikChange® Site-Directed Mutagenesis Kit. Genes carrying the desired mutations were expressed in *E. coli* BL21 (DE3) cultured in LB medium, as described above. The kinetic constants towards MP of purified enzyme and its mutants were determined using the method described above.

2.6. Homology modeling and substrate docking

The 3D structures of *Bj*MPH and *Bj*MPH$_{T64N}$ were modeled using the SWISS-MODEL server (http://swissmodel.expasy.org/) with subunit B of *Ps*MPH as the template (PDB ID: 1P9E_b). The residues Val31, Leu33, Phe85, Trp145, Phe162, Leu224 and Leu239 of *Bj*MPH were selected as the substrate binding pocket [19]. Hydrogen-bonding interactions of the 64th residue in *Bj*MPH and mutant *Bj*MPH$_{T64N}$ were identified using Pymol. An automated docking program AutoDock Vina was used for the docking of methyl-parathion into the model structure of *Bj*MPH or its mutant. A grid box of 20 × 20 × 20 with a spacing of 1.0 Å was centered in the active site as the search space to search the suitable substrate bind-