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Research article

A novel chlorpyrifos hydrolase CPD from *Paracoccus* sp. TRP: Molecular cloning, characterization and catalytic mechanism

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ABSTRACT

Background: Biodegradation is a reliable approach for efficiently eliminating persistent pollutants such as chlorpyrifos. Despite many bacteria or fungi isolated from contaminate environment and capable of degrading chlorpyrifos, limited enzymes responsible for its degradation have been identified, let alone the catalytic mechanism of the enzymes.
Results: In present study, the gene *cpd* encoding a chlorpyrifos hydrolase was cloned by analysis of genomic sequence of *Paracoccus* sp. TRP. Phylogenetic analysis and BLAST indicated that CPD was a novel esterase of organophosphate hydrolases. The purified CPD enzyme, with conserved catalytic triad (Ser155-Asp251-His281) and motif Gly-Asp-Ser-Ala-Gly, was significantly inhibited by PMSF, a serine modifier. Molecular docking between CPD and chlorpyrifos showed that Ser155 was adjacent to chlorpyrifos, which indicated that Ser155 may be the active amino acid involved in chlorpyrifos degradation. This speculation was confirmed by site-directed mutagenesis of Ser155Ala accounting for the decreased activity of CPD towards chlorpyrifos. According to the key role of Ser155 in chlorpyrifos and molecular docking conformation, the nucleophilic catalytic mechanism for chlorpyrifos degradation by CPD was proposed.
Conclusion: The novel enzyme CPD was capable of hydrolyze chlorpyrifos and Ser155 played key role during degradation of chlorpyrifos.

1. Introduction

Chlorpyrifos (O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate) has been extensively applied to agricultural and household pest control since 1965. The excessive use and persistent property of chlorpyrifos have caused numerous environmental concerns such as residues on agricultural products, contamination of soils and aquatic environments. Chlorpyrifos ultimately poses adverse effecd on non-target vertebrates mainly by inhibiting the activity of acetylcholin esterase, and overexposure of it can result in acute neurotoxicity, consequently convulsion, paralysis and death [1]. In addition, TCP (3,5,6-trichloro-2-pyridinol), the degradation product of chlorpyrifos, may be more recalcitrant than the parent compound because only a minority of chlorpyrifos-degrading microorganisms can metabolize TCP [2,3,4,5,6]. Thus, it is urgent to search for strategies for detoxifying or completely degrading chlorpyrifos from the contaminated environment.

Abiotic degradation of chlorpyrifos, such as chemical treatment, photodecomposition, volatilization and incineration, is always inefficient, costly and environmentally hazardous [7]. Biodegradation, caused by key enzymes of microorganisms, has been regarded as a reliable approach to remove chlorpyrifos effectively and completely. To date, several chlorpyrifos degraders, including bacterial and fungal strains, have been successively isolated and characterized, such as *Enterobacter* sp. B-14 [8], *Stenotrophomonas* sp. YC-1 [9], *Sphingomonas* sp. Dsp-2 [10], *Lactobacillus brevis* WCP902 [11], *Synechocystis* sp. PUPCCC 64 [12], *Stenotrophomonas maltophilia* MHF ENV20 [13], *Cupriavidus* sp. DT-1 [5], *Ochrobactrum* sp. JAS2 [2] and *Cladosporium cladosporioides* Hu-01 [4]. However, only a few genes and corresponding enzymes involved in chlorpyrifos degradation are identified. The enzyme MPH encoded by *mpd* gene was commonly reported to be able to hydrolyze chlorpyrifos to TCP [5,9,10,13]. A novel esterase OpdB from *Lactobacillus brevis* WCP902 can also hydrolyze chlorpyrifos efficiently [11]. The active site of OpdB was Ser82 and its optimal condition for chlorpyrifos hydrolysis was partial

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acid, different from the general esterase preferring to neutral to alkali environment. Park et al. purified a novel thermostable arylesterase from the archaeon *Sulfolobus solfataricus* P1 [14], which exhibited not only carboxylesterase activity, but also paraoxonase activity towards organophosphates. The chlorpyrifos-hydrolyzing enzyme CPH was identified from fungus *Cladosporium cladosporioides* Hu-01 [15], which required no cofactors for chlorpyrifos degradation and was strongly inhibited by Hg^{2+}, Fe^{3+}, DTT, β-mercaptoethanol and SDS. All of these enzymes from diverse strains account for the chlorpyrifos degradation, suggesting that there may be some features in common such as the similar structure of active domains.

Paracoccus is a metabolically versatile genus with diverse capabilities of degradation [16], including several pesticide-degrading strains isolated from contaminated environments and applied in biodegradation and bioremediation [6,17,18,19]. *Paracoccus* sp. TRP, a strain isolated from activated sludge of a pesticide plant, is capable of degrading chlorpyrifos effectively [6]. The genomic sequence of TRP has been reported, from which several groups of genes for xenobiotic biodegradation and metabolism have been predicted [20]. In present study, a novel gene cpd encoding chlorpyrifos hydrolase was identified in Paracoccus genus for the first time, which extended the host bacteria of chlorpyrifos hydrolase. The CPD enzyme was capable of degrading chlorpyrifos efficiently and would be a strong candidate for bioremediation of chlorpyrifos-contaminating environment. Molecular docking and site-directed mutation revealed the key role of Ser155 during catalysis against chlorpyrifos. Moreover, the deduced nucleophilic catalysis mediated by Ser155 laid the foundation for investigation of molecular mechanism of chlorpyrifos hydrolysis.

2. Materials and methods

2.1. Chemicals and strains

Chlorpyrifos was obtained from Shandong Tiancheng Biological Technology Co., Ltd. Stock solution of chlorpyrifos (20 g/L) was prepared in methanol. Blocking buffer, primary antibody (mouse-anti-His antibody), secondary antibody (goat-anti-mouse IgG/HRP-conjugate), and color development solution 3,3′,5,5′-Tetramethylbenzidine(TMB)were purchased from TIANGEN Biotech (Beijing) Co., Ltd. Ni-NTA fast start kit was purchased from QIAGEN China (Shanghai) Co., Ltd. All the other reagents used were of analytical grade. Basal salts medium (BM) contained (g/L) NH_4NO_3 (1.0), $MgSO_4 \cdot 7H_2O$ (0.5), $(NH_4)_2SO_4$ (0.5), KH_2PO_4 (0.5), NaCl (0.5), and $K_2HPO_4 \cdot 3H_2O$ (1.5). The following bacterial strains were used: *Paracoccus* sp. TRP and *Escherichia coli* BL21 (DE3) (TIANGEN). The expressing vector pET-32a (+) (Novagen) was employed.

2.2. Degradation of chlorpyrifos by rejuvenated Paracoccus sp. TRP

Paracoccus sp. TRP was rejuvenated by several rounds of cultivation stressed by chlorpyrifos, and the culture was diluted and spread on BM plate containing 50 mg/L of chlorpyrifos. The colony with rapid growth was picked and cultivated in LB medium. When the culture reached logarithmic phase, it was centrifuged, washed with sterile ddH_2O twice and suspended in ddH_2O. The suspended cells were inoculated into 10 mL of BM medium containing 50 mg/L of chlorpyrifos with OD_{600} = ~0.02, and cultivated at 200 rpm and 30°C for 7 d. The same volume of ddH_2O instead of inoculants was used in a control. After sampling every day during cultivation, OD_{600} of the bacterial suspension was measured, and the residual chlorpyrifos was detected by gas chromatography. The experiments were independently conducted in triplicate.

2.3. Gene cloning and expression of CPD

The *cpd* gene of putative esterase was cloned by analysis of genomic sequence of TRP and the prediction of ORFs. The conserved domain of deduced protein CPD was predicted based on conserved domain database (CDD) of NCBI. The signal sequence was predicted by SignalP-4.0. The phylogenetic tree was constructed by MEGA 6.0. To amplify the *cpd* gene excluding stop codon (NCBI Reference Sequence: NZ_AEPN01000060.1; region 6585 to 7511), the forward primer (5′-CCGGAATTCATGACCAACGCCGGTT CCCT-3′, the EcoR I site underlined) and reverse primer (5′-ACGCGTCGACGGTCATTGCG CGAAAGGCCT-3′, the Sal I site underlined) were designed. The PCR products were digested by EcoR I and Sal I, and inserted into the pET-32a(+) vector at the corresponding sites. The resulting plasmid pET-*cpd* was transformed into *E. coli* BL21 (DE3). The transformants were spread on LB plate with 1% tributyrin, and the positive clone was determined by sequencing. The positive clone was cultivated in LB medium with 100 μg/mL ampicillin at 37°C and 180 rpm to an OD_{600} of 0.6, when 1 mM of IPTG was added. After induction for 3 h, the cells were harvested by centrifugation at 12000 rpm and 4°C for 10 min. The *E. coli* BL21 (DE3) with pET-32a (+) was applied as control. The total proteins were analyzed by SDS-PAGE followed by western blot. The proteins were transferred onto nitrocellulose membrane which were sequentially blocked by BSA solution, incubated with primary antibody (mouse-anti-His antibody, 1:2000 dilutions) and secondary antibody (goat-anti-mouse IgG/HRP-conjugate, 1:1000 dilutions), and finally developed with TMB solution. The recombinant CPD was purified under native conditions according to QIAexpress Ni-NTA fast start handbook and its concentration was measured using bovine serum albumin as standard protein according to the method developed by Lowry et al. [21].

2.4. Chlorpyrifos degradation by the CPD enzyme

To investigate the chlorpyrifos degrading capability of CPD, 5 mL of PBS medium containing 50 mg/L chlorpyrifos and appropriate amount of recombinant CPD was incubated at 30°C for 15 min. The residual chlorpyrifos was measured by gas chromatography. Three independent experiments were performed. Under the same conditions, controls were examined in the absence of CPD. One unit (U) of enzyme activity was defined as the amount of the recombinant CPD required to hydrolyze 1 μmol chlorpyrifos per min at 30°C. The data were reported as specific activity (U/mg proteins). The activity of mutant CPD protein was determined using the same method.

2.5. Characterization of CPD

The activity assay was carried out in 2 mL of PBS with 0.3 mM α-naphthyl acetate and appropriate amount of recombinant CPD. After incubated at 30°C for 15 min, 200 μL of TMB solution was added into the reaction mixture. When the dark cyan appeared and was stable, OD_{600} of the mixture was detected. PBS solution was applied instead of the CPD in a control. The assay experiment was performed in triplicate independently. The effects of potential activators or inhibitors on enzymatic activity were investigated, including metal ions (1 mM Mn^{2+}, Mg^{2+}, Zn^{2+}, Co^{2+} and Ca^{2+}), and chemical agents EDTA (1 mM), PMSF (1 mM), DEPC (1 mM), Tween-20 (0.025%) and SDS (0.025%). The residual activity was detected as described above, and expressed as percentage of enzymatic activity of the control without any additives above.

2.6. Molecular docking

The homologous modeling of CPD was conducted using the automated mode in SWISS-MODEL (http://swissmodel.expasy.org/), and extremely thermophilic esterase Pest E (PDB ID: 3zwqA, resolution: 2.00 Å, 313Aa) from *Pyrobaculum calidifontis* VA1 was selected as a template [22]. Molecular graphics were performed using the UCSF Chimera package [23].The molecular interaction between the CPD enzyme and chlorpyrifos was simulated using EADock DSS

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