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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 contaminated 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 member 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 degradation 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 were 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-contaminated 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 CGAA AGGCCT-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 was 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 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 an 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

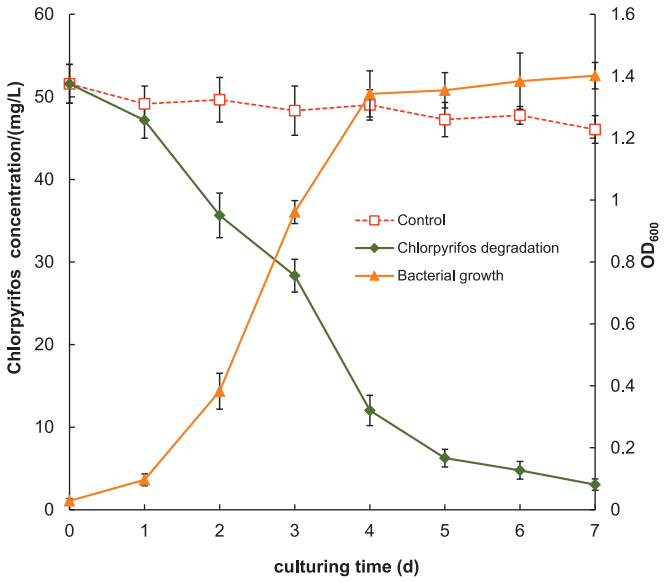

Fig. 1. Growth curve and chlorpyrifos degradation kinetics of strain TRP.

program in SwissDock (http://swissdock.vital-it.ch/docking). According to the FullFitness values, the molecular docking results were ranked and clustered [24].

2.7. Site-directed mutagenesis of the cpd gene

Ser155Ala mutation of the CPD enzyme was performed by site-directed mutagenesis technology (TaKaRa MutanBEST Kit). The primers 5′-TCCGGCGACGCTGCCGGTGCCAATCT-3′ (forward) and 5′-AATCGACCATTTGCGTTCGGGCAAAAT-3′ (reverse) were designed based on the *cpd* gene sequence. The change of the codon from AGT to GCT in the *cpd* gene led to the Ser155Ala mutation in the CPD enzyme. Complementary base of the 5′-terminus of reverse primer was adjacent to the 5′-terminus base of the forward primer in the template pET-*cpd*. The purified and self-ligated PCR products were transformed into *E. coli* BL21 (DE3), which were spread on LB plate containing 100 μg/mL ampicillin and cultivated at 37°C for 16 h. The positive plasmid was verified by sequencing and designated as pET-*cpd*-S155A.

2.8. Analytical methods

The residual chlorpyrifos of culture medium or reaction mixture was extracted by the same volume of n-hexane followed by gas chromatography (GC-2010 system, Shimadzu). The conditions for GC analysis were as follows: the injector temperature was 300°C, the column was initially equilibrated at 150°C for 3 min, the column temperature was held at 150°C for 2 min, programmed to 270°C at 8°C per min and held at 270°C for 5 min, and the detector temperature was 330°C. The carrier gas nitrogen was injected at a flow rate of 1 mL/min and with the pressure of 97.7 kPa. 1 μL of sample was injected with a split ratio of 19:1 [25].

3. Results

3.1. Degradation of chlorpyrifos by rejuvenated Paracoccus sp. TRP

The preserved strain *Paracoccus* sp. TRP was rejuvenated in order to recover the degradation capability, and one clone was chosen for further study based on its rapid growth on the MSM plate containing chlorpyrifos. The clone could rapidly degrade 50 mg/L of chlorpyrifos from the 2nd day to the 4th day during the logarithmic phase of the bacterial growth. The degradation rate gradually slowed down with the decreased bacterial growth rate after the 4th day. Approximately 83.3% of chlorpyrifos was removed at the 7th day and less than 5% in the control, indicating that the strain TRP played a leading role in chlorpyrifos degradation (Fig. 1). The results demonstrated that the degradation ability of the strain TRP had recovered to the greatest extent after the rejuvenation, and this strain could serve in remediation of chlorpyrifos-contaminated environment.

3.2. Gene identification and sequence analysis of CPD

To explore the hydrolase involved in chlorpyrifos degradation, the whole genome of *Paracoccus* sp. TRP was sequenced. Based on BLASTP program (protein–protein BLAST (Basic Local Alignment Search Tool) program) with the organophosphate hydrolases as queries [10,11,14,26,27,28,29,30,31,32,33,34], a putative esterase/lipase/thioesterase (NCBI Reference Sequence: **WP_010400062.1**) was identified with the most similarity among the deduced proteins of TRP. The esterase (designated as CPD) shared 24.5% identity with chlorpyrifos hydrolase OpdB from *Lactobacillus brevis* WCP902 [11], and 23.4% with an arylesterase effective on organophosphate from *Sulfolobus solfataricus*

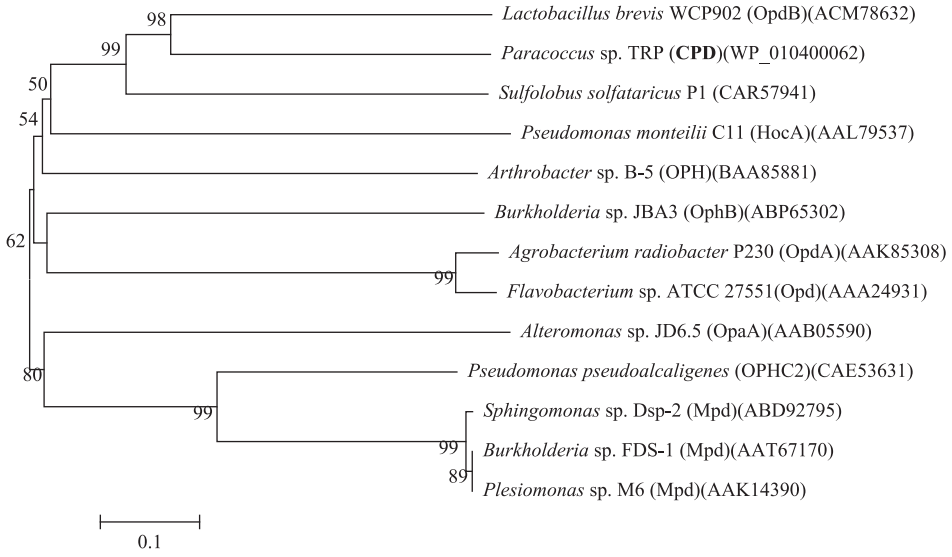

Fig. 2. Phylogenetic tree of organophosphate-degrading enzymes constructed by the neighbor-joining method. The names and accession numbers of hydrolases followed the strain.

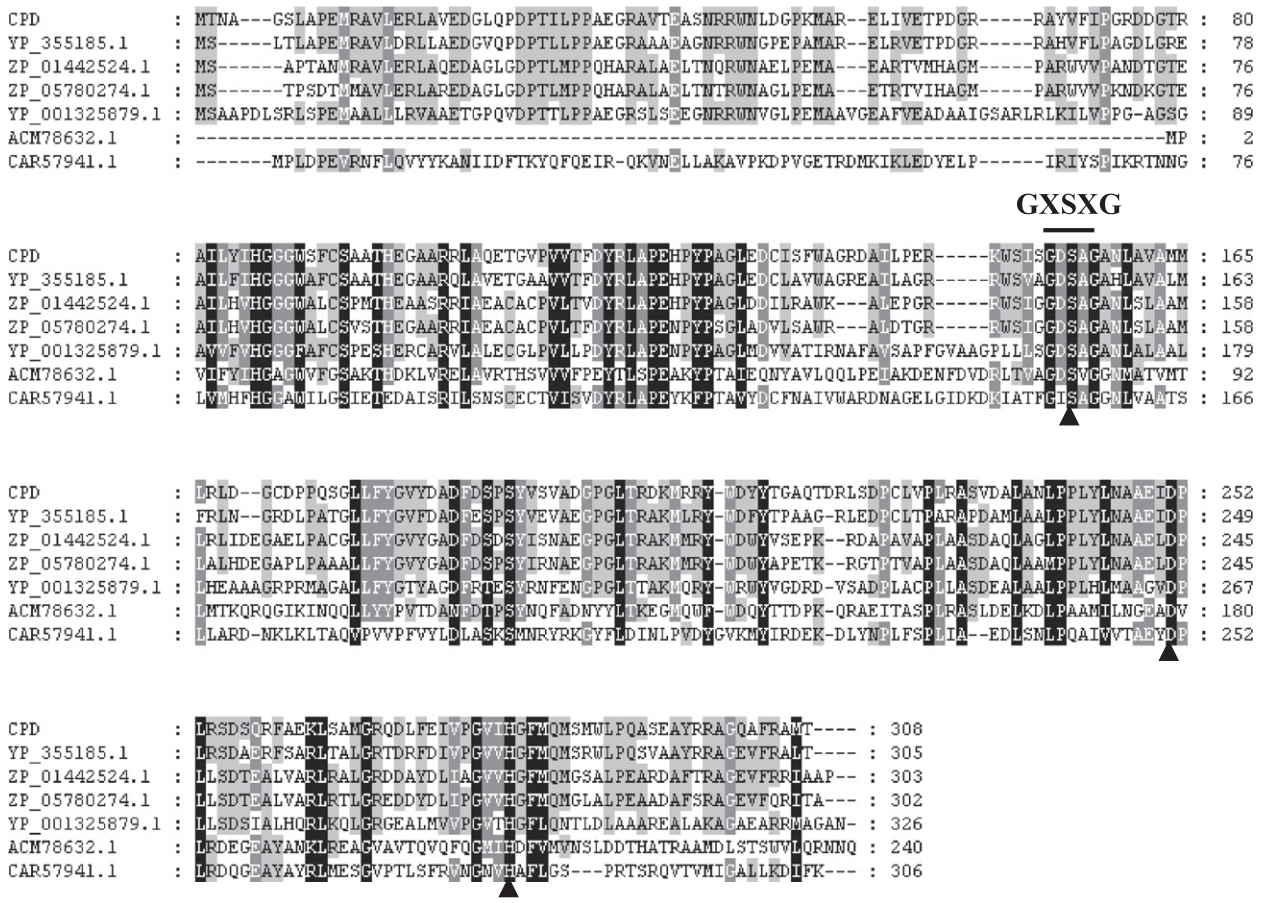

Fig. 3. Multiple sequence alignments of esterases from *Paracoccus*sp. TRP (CPD) (WP_010400062), *Rhodobactersphaeroides*(YP_355185), *Pelagibacabermudensis* (ZP_01442524), *Citreicella*sp.SE45 (ZP_05780274), *Sinorhizobiummedicae*WSM419 (YP_001325879), *Lactobacillus brevis*WCP902 (OpdB) (ACM78632), *Sulfolobus solfataricus* P1 (CAR57941). The catalytic triad (Ser155, Asp251, and His281) is indicated as the solid triangle. The consensus sequence motif Gly-X-Ser-X-Gly around the active site Ser, is shown above the aligned sequences.

P1 [14]. Therefore, the esterase CPD may be responsible for chlorpyrifos hydrolysis. From the phylogenetic tree built by CPD and other organophosphate hydrolases (Fig. 2), the CPD protein was closely clustered with OpdB of *Lactobacillus brevis* WCP902 [11] and the arylesterase of *Sulfolobus solfataricus* P1 [14], indicating that it may be a novel member of organophosphate hydrolases. The CPD protein was composed of 308 amino acids with a calculated molecular mass of 33.86 kDa. There was no signal sequence according to the amino acid sequence analysis by SignalP-4.0. Based on conserved domain search, CPD had α/β hydrolase domain and a substrate binding pocket ILYIHGGGWSFCSA which may contribute to interaction with chlorpyrifos. The esterase had the motif Gly-X-Ser-X-Gly (Gly153-Asp-Ser-Ala-Gly157) and putative catalytic triad Ser155-Asp251-His281 (Fig. 3), which were generally conserved in hydrolases. According to these results above, the CPD protein was predicted to be a novel chlorpyrifos hydrolase.

3.3. Purification and degradation capability of CPD

To determine the chlorpyrifos hydrolysis activity of CPD, the recombinant CDP protein encoded by plasmid pET-*cpd* was expressed in *E. coli* BL21 (DE3). Most of transformed *E. coli* BL21 (DE3) were surrounded by transparent halo on the plate, indicating that the CPD has esterase activity. On sodium dodecyl sulfate-polyacrylamide gel, there was an obvious band of approximately 50 kDa for induced positive clone (Fig. S1A), consistent with the calculated molecular weight of recombinant CPD (51.7 kDa). Western blot showed a blue band at the position of the protein of 50 kDa (Fig. S1B), which confirmed the identity of recombinant CPD. The recombinant CPD was

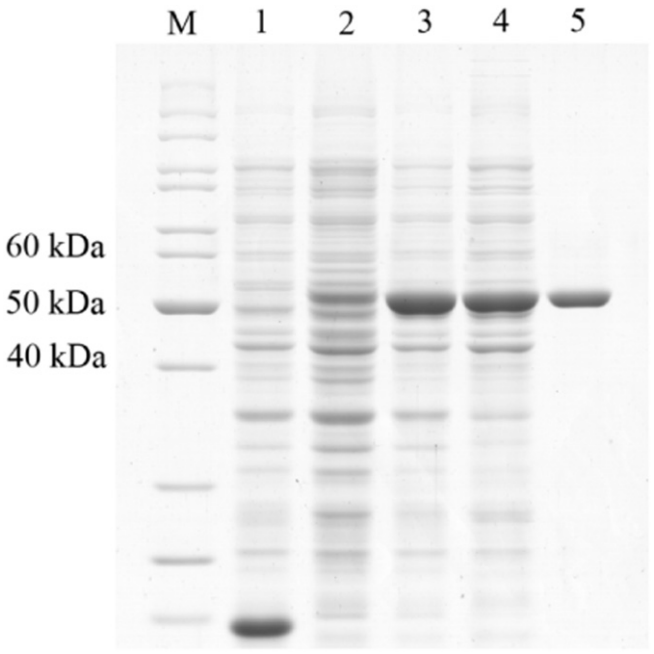

Fig. 4. Analysis of recombinant CPD expressed in *E. coli* BL21(DE3) on SDS-PAGE. M, molecular mass markers; lane 1, pET-32a(+) induced by IPTG for 2 h; lane 2, pET-*cpd* not induced; lane 3, pET-*cpd* induced by IPTG for 3 h; lane 4, supernatant of induced pET-*cpd*; lane 5, purified recombinant CPD.

Table 1
Effects of metal ions and chemical agents on the activity of CPD.

Reagent	Concentration	Residual activity ± SD (%)
Control	0 mM	100 ± 0.00
Mn^{2+}	1 mM	95.57 ± 4.15
Mg^{2+}	1 mM	95.38 ± 3.26
Zn^{2+}	1 mM	91.20 ± 2.52
Co^{2+}	1 mM	92.34 ± 3.05
Ca^{2+}	1 mM	87.03 ± 1.97
Hg^{2+}	1 mM	53.33 ± 10.70
EDTA	1 mM	96.56 ± 4.42
PMSF	1 mM	69.95 ± 1.34
DEPC	1 mM	95.64 ± 2.58
Tween-20	0.25 mg/mL	106.49 ± 1.02
SDS	0.25 mg/mL	19.77 ± 0.34

purified to electrophoretic homogeneity by Ni^{2+} column (Fig. 4). When the CPD was incubated with 50 mg/L chlorpyrifos in PBS for 15 min, 63.5% ± 2.14% of chlorpyrifos was removed with the enzyme activity of 59.62 U/mg proteins. This finding revealed that the *cpd* gene encoded a novel chlorpyrifos-degrading enzyme. Interestingly, the different genes from various microbes could degrade organophosphate [11,14,29,35], and the identical gene, such as *mpd*, also existed in diverse strains from a variety of geographical areas [5,9,10]. It suggested that the genes of organophosphate hydrolases may be divergent from different ancestors and horizontal gene transfer (HGT) may be critical role in the distribution of the gene.

3.4. Effects of metal ions and chemical agents on the enzymatic activity

The CPD enzyme is one member of esterase/lipase superfamily, and the enzyme activity towards α-NA, the standard esterase substrate, was investigated. The reaction mixture appeared aquamarine blue in the experimental group with CPD protein and α-NA, while the solution appeared tan in the control, suggesting that the CPD enzyme had esterase activity to α-NA. The effects of metal ions and chemicals on

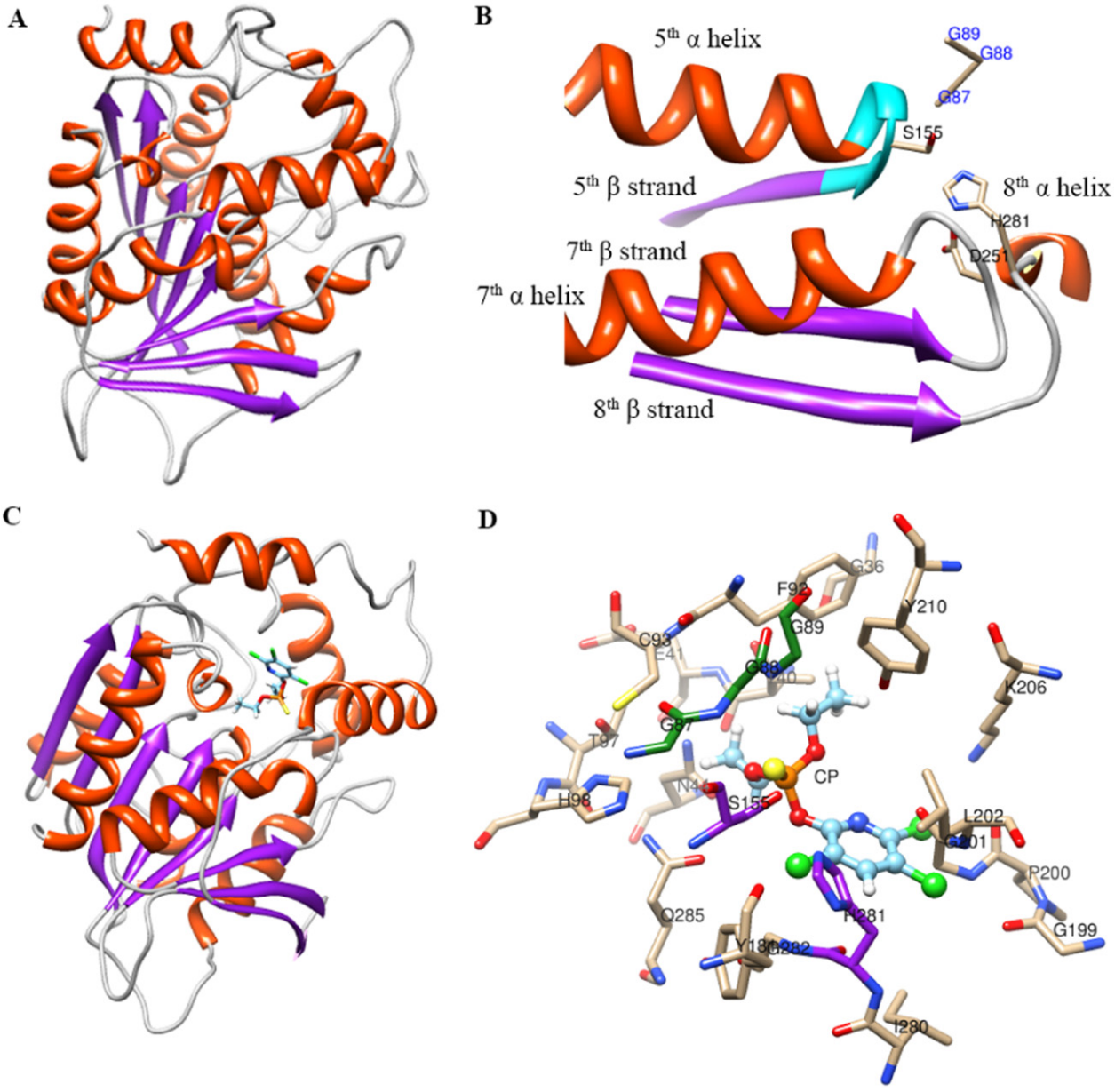

Fig. 5. Structure of the CPD enzyme and molecular docking of CPD and chlopyrifos. (A) Overall 3D structure of the CPD enzyme with secondary structural elements colored. (B) Views of the catalytic triad (Ser155, Asp251 and His281), and substrate binding pocket (ILYIHGGGWSFCSA). (C) Optimal molecular docking result between the CPD and chlorpyrifos. (D) Residues distribution around the chlorpyrifos molecule within 5 Å. Ser155 and His281 were colored in purple and Gly87-Gly88-Gly89 green.

the esterase activity of the CPD were shown in Table 1. The activity of the CPD enzyme was significantly inhibited by Hg^{2+}, but not severely affected by Mn^{2+}, Mg^{2+}, Zn^{2+}, Co^{2+}, Ca^{2+} and chelating agent EDTA. This finding indicated that the CPD protein contained no metal ions, or the esterase activity did not require the metal ions tested in this study, which was in agreement with the finding that CPH from fungus *C. cladosporioides* Hu-01 [15]. However, phosphotriesterases from *Pseudomonas diminuta* MG contained a single Zn^{2+} [36], and OPAA from *Alteromonas* sp. JD 6.5 was stimulated by Mn^{2+} and Co^{2+} [30]. Ionic detergent SDS caused a sharp decrease of the enzymatic activity by 80% because of its serious damage to the structure of protein. The enzyme activity of CPD dropped greatly in the presence of PMSF, a serine protease inhibitor, which led to reasonable deduction that the serine of CPD was of importance for catalysis and Ser155 of catalytic triad may be the causal residue. In addition, the histidine modifier DEPC caused slight decrease of activity, suggesting that histidine may be also a critical residue.

3.5. Molecular docking and catalytic mechanism

According to the three-dimensional structure model of CPD built by homologous modeling, CPD had a tightly folded globular structure consisting of nine α helixes, eight β strands, and several coils (Fig. 5A). The residue Ser155 of the putative active site located at the loop between the fifth β strand and the fifth α helix from N terminus. This flexible region may facilitate its interaction with substrate. In addition, the other two residues Asp251 and His281 in putative catalytic triad also located at strongly flexible loops: Asp251 at the loop between the seventh β strand and the seventh α helix, and His281 at the loop between the eighth β strand and the eighth α helix (Fig. 5B).

Based on the molecular docking between CPD and chlorpyrifos (Fig. 5C), the putative chlorpyrifos binding sites (Gly87-Gly88-Gly89) and two critical amino acids (Ser155 and His281) of the catalytic triad presented in the area 5 Å from chlorpyrifos (Fig. 5D). The distance between O-atom of hydroxyl group from Ser155 and P-atom from chlorpyrifos was 6.062 Å, which may be beneficial to the initial nucleophilic attack on P-atom by O-atom, causing the major product TCP to be released. Sequentially, P-atom from chlorpyrifos was attacked by O-atom from water, which resulted in another product DETP. Simultaneously Ser155 returned to its original state. This might be the catalytic mechanism for the first step of chlorpyrifos degradation by CPD to TCP and DETP. Thus, Ser155 might be the crucial amino acid for enzyme activity. The similar role of serine had already been previously studied. For instance, in *Lactobacillus brevis* WCP902, the active site of OpdB for chlorpyrifos hydrolysis was Ser82 [11], and the arylesterase from *Sulfolobus solfataricus* P1 was a serine esterase and contained the active site Ser156 [14]. His281 of the catalytic triad also located within the area of approximately 5 Å from chlorpyrifos molecule, but Asp251 located outside the area, indicating that His281 might be also involved in chlorpyrifos degradation (Fig. 5D).

3.6. Effects of Ser155Ala mutation on degradation capability

According to molecular docking and the inhibitory effect, Ser155 was supposed to contribute greatly to chlorpyrifos degradation. To confirm this speculation, Ser155Ala mutation was performed by site-directed mutagenesis technology. An expression vector named pET-*cpd*-S155A was constructed and the mutant CPD protein was expressed and purified successfully (Fig. S2). Only 5.15% ± 1.03% of 50 mg/L chlorpyrifos was degraded by the mutant CPD enzyme within 15 min and the enzymatic activity was 7.85 U/mg proteins. The degradation percentage of chlorpyrifos was reduced by more than 10 times when the mutation of Ser155Ala was introduced, strongly demonstrating that Ser155 was a key residue and may be involved in nucleophilic catalysis.

Conflict of interest

None.

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Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejbt.2017.10.009

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