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Molecular cloning, purification and biochemical characterization of a novel pyrethroid-hydrolyzing carboxylesterase gene from *Ochrobactrum anthropi* YZ-1

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ABSTRACT

Strain YZ-1 was isolated from activated sludge and identified as *Ochrobactrum anthropi*. This strain was capable of degrading pyrethroids pesticides, suggesting the presence of degrading enzymes. In the present study, a novel esterase gene *pytZ* was cloned from the genomic library of YZ-1 successfully. The *pytZ* contained an open reading frame of 606 bp encoding a pyrethroid-hydrolyzing carboxylesterase. Deduced amino acid sequence showed moderate identities (39–59%) with most homologous carboxylesterase, except a putative carboxylesterase from *O. anthropi* ATCC 49188 with the highest identity of 85%. Phylogenetic analysis revealed that PytZ belonged to esterase VI family. The gene *pytZ* showed no any sequence similarity with reported pyrethroid-hydrolyzing genes and was a new pyrethroid-degrading gene. PytZ was expressed in *Escherichia coli* BL21 (DE3) and purified using Ni-NTA Fast Start. PytZ was able to degrade various pyrethroids. The optimal temperature and pH were 35 °C and 7.5. This enzyme was very stable over a wide range of temperature and pH. No cofactors were required for enzyme activity. Broad substrate specificity, high enzyme activity, and the favorable stability make the PytZ a potential candidate for the detoxification of pyrethroid residues in biotechnological application.

1. Introduction

Pyrethroids are mostly used insecticides throughout the world. They are synthetic analogs of the natural pyrethrin, which is one kind of toxin with insecticidal activity deriving from the flowers of *Chrysanthemum cinerariaefolium*. Pyrethroids were classified as type I or type II based on the toxic symptoms and the absence or the presence of a cyano group at the carboxyl alpha position [1]. Pyrethroid pesticides have been used for more than 30 years, and accounted for more than 30% of insecticide market [2]. With the withdrawal of organophosphorus insecticide from the market, their usage is continuing to grow.

Pyrethroid pesticides are widely used for their high insecticidal activity and were generally considered low toxic for mammalian [3]. However, extensive application of this kind of pesticides has caused many problems, such as pest resistance, soil and water contamination, high residue in agricultural product, and human exposure. Previous studies reported that the high exposure to pyrethroids might cause reproductive toxicity [4], cytotoxicity [5,6], and adverse effects on many tissues [7,8]. Some of them have been classified as a possible human carcinogen by the Environmental Protection Agency (EPA) of US [9,10]. In addition, most pyrethroid insecticides possess acute toxicity to some no-target organisms, such as been, silkworm, especially aquatic invertebrates, often at the concentration less than 1 $\mu g\,L^{-1}$ [11,12]. These findings revealed that pyrethroids are potentially harmful to human health and ecosystem. Great concerns have been raised about the pyrethroid residues and persistence in the environment. Therefore, it is urgent to develop some efficient strategies to solve these problems caused by pyrethroid residues.

In natural environment, pyrethroid pesticides are degraded mainly by photolysis, hydrolysis and microbial decomposition [13]. Microbial degradation plays an important role in the elimination of pyrethroid residues. Biodegradation is an important environment biotechnology for the treatment of organic pollutants. One strategy of which is using some key enzymes to break down pesticide residues. For pyrethroid pesticides, they are a large class of compounds that contain an ester bond structure in molecular, which was formed by an alcohol and acid moieties. The major metabolic pathway of pyrethroids in resistant insects and degrading microorganisms involves in oxidation by cytochrome P450s and hydrolysis by esterases [14]. Carboxylesterases are subtypes of esterases and included within subgroup 3.1.1 of the International Union of Biochemistry. They are able to hydrolyze a large number of ester-containing compounds, such as carbamates and pyrethroids [15]. It has been confirmed that carboxylesterase is responsible for the detoxification of pyrethroids by hydrolyzing the ester bond [16]. Many pyrethroid-degrading strains have been isolated, and degradation studies were also carried out [9,10,17–19].

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Some pyrethroid-degrading carboxylesterases were purified and characterized from pyrethroid-degrading microorganisms [14–16], animal liver microsomes [20,21] and pyrethroid-resistant insects [22]. However, the reports about pyrethroid-degrading genes are still rare. So far, only several degrading genes were cloned, i.e. pyrethroid-hydrolyzing genes from *Klebsiella* sp. ZD112, *Sphingobium* sp. JZ-1 and metagenome [23–25].

In this study, we mentioned the isolation and identification of a pyrethroid-degrading strain *Ochrobactrum anthropi* YZ-1, the cloning and expression of the gene *pytZ* which encoded a novel pyrethroid-hydrolyzing carboxylesterase, and the characterization of the purified enzyme, such as substrate specificity, stability, optimal temperature and pH.

2. Materials and methods

2.1. Strains, plasmids and media

Escherichia coli DH5α and *E. coli* BL21 (DE3) were purchased from Tiangen, and were used as cloning and expression host cell respectively. The plasmids pUC18 (Tiangen) and pET30a (+) (Novagen) were used for cloning and expression vectors accordingly. Mineral salt medium (MSM) that containing (g L^{-1}) NH_4NO_3, 1.0; NaCl, 0.5; $(NH_4)_2SO_4$, 0.5; KH_2PO_4, 0.5; and K_2HPO_4, 1.5 was used for the isolation and determination of degrading ability. Luria-Bertani (LB) medium containing (g L^{-1}) peptone, 10; yeast extract, 5.0 and NaCl, 10.0 was used for the genomic library construction and recombinant protein expression. Appropriate antibiotics were added when required.

2.2. Chemicals and enzymes

Lambda-cyhalothrin (98%), beta-cypermethin (95%), beta-cyfluthrin (96%), deltamethrin (98%) and permethrin (95%) were provided by Jiangsu Yangnong Chemical Group Co. Ltd. (Yangzhou, China). All the enzymes used were purchased form Takara. *P*-nitrophenyl esters were purchased from Sigma. All other chemicals were of analytical grade and purchased from commercial sources. Protein molecular mass marker was product of Tiangen.

2.3. Analysis condition of gas chromatography (GC)

Pyrethroid residue was extracted with equal volume *n*-hexane twice. The organic layer was filtrated, dried and re-dissolved in *n*-hexane and determined by GC. The analysis conditions were as follows: ECD, RTX-1301, carrier gas of N_2 (99.999%) at 1.0 mL min^{-1}; inlet temperature of 260 °C, temperature programming of 230 °C 8 min, increasing to 280 °C at 25 °C min^{-1} and remaining 6 min, detector temperature of 300 °C; 1 μL sample with split ratio of 49:1.

2.4. Isolation and identification of pyrethroid-degrading strains

Activated sludge was collected from an aerobic pyrethroid manufacturing wastewater treatment system. To isolate pyrethroid-degrading bacteria, 5 g of activated sludge as initial inoculum was inoculated into MSM which was only added 100 mg L^{-1} lambda-cyhalothrin as sole carbon source for microorganism growth. After six days of incubation at 30 °C and 180 rpm on a rotary shaker, 5 mL of culture was transferred into fresh MSM medium containing 200 mg L^{-1} lambda-cyhalothrin and incubated for another six days. The transfer was conducted for six times successively until the concentration of lambda-cyhalothrin in MSM gradually increased to 800 mg L^{-1}. Then the culture was diluted and spread on MSM agar plate containing 600 mg L^{-1} lambda-cyhalothrin. The target strain was selected and purified. Their degrading ability to pyrethroids was determined by GC. The strain which possessed the highest degrading ability to lambda-cyhalothrin was identified by morphological, physiological and biochemical characteristic [26], and 16S rDNA gene analysis [27].

2.5. Genomic library construction and screening

Genomic DNA extraction was conducted using TIANamp Bacteria DNA Kit (Tiangen) according to the manufacturer's instructions. To construct a size-fractionated genomic library, genomic DNA of *O. anthropi* YZ-1 was partially digested with *Sau*3AI. DNA fragments from 1 to 3 kb were pooled and ligated into the pUC18 plasmid vector which had been previously digested with *Bam*HI. The recombinant plasmids were used to transform competent cells of *E. coli* DH5α. Constructed genomic library was plated onto LB agar plates containing 100 μg mL^{-1} ampicillin. The plates were incubated at 37 °C for about 48 h. The initial screening of transformants was performed as described previously [28]. Then the obtained clones by initial screening were transferred to MSM agar plate containing 600 mg L^{-1} lambda-cyhalothrin as sole carbon scours for the second screening based on the growth status. Finally, the screened clones were tested for their degrading ability to lambda-cyhalothrin by GC.

2.6. Sequence analysis

The recombinant plasmid was extracted and sequenced. Open reading frame (ORF) search was performed by ORF Finder online tool http://www.ncbi.nlm.nih.gov/gorf/gorf.html and DNASTAR software. The searches for nucleotide and protein sequence homology were conducted with BLAST program at NCBI http://www.blast.ncbi.nlm.nih.gov/. Multiple sequence alignment was carried out using CLUSTAL W program [29]. The result was visually highlighted with BoxShade Server program http://www.ch.embnet.org/software/BOX_form.html. Phylogenetic tree was constructed using MEGA 4.0 software by neighbor-joining method. Bootstrapping (1000 replicates) was used to estimate the confidence levels of phylogenetic reconstructions [27]. All the sequences used were retrieved from GenBank and SWISS-PROT database.

2.7. Expression and purification of pyrethroid-hydrolyzing carboxylesterase

For expression of pyrethroid-hydrolyzing carboxylesterase, the complete open reading frame of *pytZ* was amplified by PCR (Peltier Thermal Cycler, Bio-Rad) using primers LF (5′-CG**GGATCC**ATGAGCGTAGTGACGATGATCGTAAAA-3′, *Bam*HI restriction site was highlighted as bold) and LR (5′-CG**GAATTC**TCAGTTGTTTTTGACGATGGGAGC-3′, *Eco*RI restriction site was highlighted as bold). PCR product was digested with *Bam*HI and *Eco*RI simultaneously. Purified target fragment was inserted into pET30a (+) expression vector which had been previously digested with the same restriction enzymes. The recombinant was used to transform *E. coli* BL21 (DE3) cells. The expression procedure was carried out according to the standard method [30]. *E. coli* BL21 (DE3) containing the pET30a-*pytZ* plasmid was inoculated in 30 mL LB medium supplemented with 50 μg mL^{-1} kanamycin and incubated at 37 °C and 180 rpm on a rotary shaker for 12 h. Culture was transferred into fresh LB medium in the proportion of 1:100 and incubated for 2 h. Then isopropyl-β-D-thiogalactopyranoside (IPTG) was added with a final concentration of 1 mM. After incubation at 30 °C for another 3 h, cells were harvested by centrifugation at 10,000 × *g* for 10 min at 4 °C. Then the protein was purified using Ni-NTA Fast Start Kit (Qiagen) according to the manufacturer's instructions. The purified protein was analyzed by SDS-PAGE.