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2,4-Dichlorophenol hydroxylase for chlorophenol removal: Substrate specificity and catalytic activity

Hejun Ren[a], Qingchao Li[b], Yang Zhan[b], Xuexun Fang[b], Dahai Yu[b,*]

[a] Key Laboratory of Ground Water Resources and Environment of the Ministry of Education, College of Environment and Resources, Jilin University, 2519 Jiefang Road, Changchun 130021, PR China
[b] Key Laboratory for Molecular Enzymology and Engineering of Ministry of Education, College of Life Science, Jilin University, 2699 Qianjin Street, Changchun 130012, PR China

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ABSTRACT

Chlorophenols (CPs) are common environmental pollutants. As such, different treatments have been assessed to facilitate their removal. In this study, 2,4-dichlorophenol (2,4-DCP) hydroxylase was used to systematically investigate the activity and removal ability of 19CP congeners at 25 and 0 °C. Results demonstrated that 2,4-DCP hydroxylase exhibited a broad substrate specificity to CPs. The activities of 2,4-DCP hydroxylase against specific CP congeners, including 3-CP, 2,3,6-trichlorophenol, 2-CP, and 2,3-DCP, were higher than those against 2,4-DCP, which is the preferred substrate of previously reported 2,4-DCP hydroxylase. To verify whether cofactors are necessary to promote hydroxylase activity against CP congeners, we added FAD and found that the added FAD induced a 1.33-fold to 5.13-fold significant increase in hydroxylase activity against different CP congeners. The metabolic pathways of the CP degradation in the enzymatic hydroxylation step were preliminarily proposed on the basis of the analyses of the enzymatic activities against 19CP congeners. We found that the high activity and removal rate of 2,4-DCP hydroxylase against CPs at 0 °C enhance the low-temperature-adaptability of this enzyme to the CP congeners; as such, the proposed removal process may be applied to biochemical, bioremediation, and industrial processes, particularly in cold environments.

1. Introduction

Chlorophenols (CPs) are a large group of chemicals with chlorine atoms (between one and five) attached to the phenolic structure; 19 congeners exist [1]. CPs are extensively used to manufacture dyes, drugs, pesticides, and other industrial products; however, these substances have been considered as a cause of global environmental concern because of their persistence, toxicity, and health risks [2]. Several physicochemical methods, including adsorption, mixing coagulation, extraction, photochemical oxidation, supersonic chemistry process, hydrogenolysis, and radiolysis, have been used to remove CPs from the environment. Physicochemical techniques provide several disadvantages, such as incomplete pollutant degradation, high costs, and additional treatments; thus, these techniques are less competitive than biological methods [3].

Biological methods, such as enzymatic and microbial biodegradation or transformation of CPs, have been extensively investigated. These methods are regarded as key remediation and environmentally friendly options that can be utilized to solve environmental pollution problems caused by these notorious compounds [4–6]. Enzymatic degradation is superior to microbial biodegradation method mainly because enzymes can tolerate concentrated CPs. The isolated enzymes are more efficient than microorganisms; their storage and handling are easier than those of microorganisms [3].

Several enzymes belonging to oxidoreductases, such as 2,4-dichlorophenol hydroxylase (2,4-DCP hydroxylase; EC 1.14.13.20), peroxidase (EC 1.11.1.7), and laccase (EC 1.10.3.2), have been utilized to remove CPs through hydroxylation [7]. Enzymatic hydroxylation is usually the initial step in the oxidative degradation pathway of CPs. This step is extremely important in CP detoxification because the produced catechol, halogenated catechols, and other derivatives after hydroxylation occurs are more readily converted to linear aliphatic compounds by the subsequent aromatic ring cleavage reaction [8].

2,4-DCP hydroxylase can be effectively used to remove CPs in the hydroxylation step; the obtained products are less toxic and easily degradable during the subsequent enzymatic reaction [9].

* Corresponding author. Fax: +86 431 85155240.
E-mail address: yudahai@jlu.edu.cn (D. Yu).

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2,4-DCP hydroxylase is a key enzyme in the pathway involved in the degradation of 2,4-dichlorophenoxyacetic acid in many bacteria [10]. In our previous study, a 2,4-DCP hydroxylase is identified in a metagenome constructed from polychlorinated biphenyl-contaminated soil, and this enzyme can be used to bioremediate 2,4-DCP because of its high hydroxylation activity and stability [11,12]. This enzyme remains active against certain CPs at low temperatures, and this ability is essential for the bioremediation of CP congeners because many pollution-related problems occur in seawaters and industrial effluents characterized by low temperatures. Therefore, cold-active enzymes instead of mesophilic enzymes can be used to remove CPs and be applied in bioremediation processes [13].

Although studies have been conducted to investigate the removal ability of partial CP congeners by using 2,4-DCP hydroxylase, few studies have been performed to elucidate the substrate specificity of this enzyme to all 19CP congeners. This study mainly aimed to extend the cold adaptability of 2,4-DCP hydroxylase to all 19CP congeners to develop new and specific degradative capabilities to bioremediate CP contamination in cold environments. This study also aimed to propose a preliminary assumption on the molecular basis of the CP degradation in enzymatic hydroxylation by comparing enzymatic activities against 19CP congeners. Cofactors, such as FAD, required for the hydroxylase activities of CP congeners were also investigated because this enzyme exhibits a high sequence and structural similarity to FAD-dependent hydroxylase [11].

2. Material and methods

2.1. Materials

CPs of analytical grade were purchased from J&K Scientific Ltd. (Shanghai, China). Methanol (high-performance liquid chromatography [HPLC] grade) was obtained from Sigma. Other chemicals of analytical grade were obtained from Sigma. Recombinant *Escherichia coli* DH5α containing the TfdB-JLU gene for 2,4-DCP hydroxylase expression was from our lab. BugBuster protein extraction reagent was from Novagen (Nottingham, UK).

2.2. Methods

2.2.1. Protein expression and purification

The recombinant *E. coli* was cultivated in LB medium containing 30 μg kanamycin/ml and 34 μg chloramphenicol/ml at 37 °C. Protein expression was induced at 18 °C by the addition of 0.2 mM isopropyl-β-D-1-thiogalactoside (IPTG) (Fisher Scientific, Fairlawn, NJ) at an OD600 of 0.4. After 15 h incubation, the cell pellets were harvested by centrifugation at 12,000 rpm and washed with 50 mM sodium phosphate buffer, pH 8.0. For the preparation of crude extract, cells (1.11 g of *E. coli* cell paste from 400 ml fermentation culture) were suspended in 4 ml pH 8.0 Bugbuster protein extraction reagent with 1 mM dithiothreitol (DTT) and 0.6 mM PMSF (phenylmethylsulfonyl fluoride) to yield a higher specific activity compared to ultrasonication. The protein extraction was performed for 10 min at 20 °C at 150 rpm. Then, the lysate was centrifuged at 12,000 rpm for 10 min using a Thermo Sorvall WX Ultracentrifuge (Fisher Scientific, Fairlawn, NJ, USA) at 4 °C. The supernatant was transferred onto a His*link*™ column (Promega, Madison, WI, USA), rinsed with wash buffer (10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 10 mM imidazole, pH 8.0), and eluted with elution buffer (10 mM HEPES, 1 M imidazole, pH 8.0). The protein supernatant was loaded onto a nickel-nitrilotriacetic agarose resin (Qiagen, Germany) equilibrated with the same buffer. After washing with 5 column volumes of the wash buffer (40 mM imidazole), the bound enzyme was eluted with the elution buffer (200 mM imidazole). The fractions containing 2,4-DCP hydroxylase activity were pooled and concentrated by ultrafiltration by using Amicon Ultra-15 centrifugal filter units (Millipore, USA) and then were diafiltered with 50 mM sodium phosphate buffer, pH 7.5, containing 10% (v/v) glycerol. A total of 4 ml protein solution (300 μg ml^{-1}) was obtained after 4000 rpm centrifugation. Samples were stored at −80 °C for further analysis.

2.2.2. Enzymatic CPs removal

The experiments on CPs removal were performed in a 500 μl eppendorf tube. The reaction mixture was placed into the air-bath constant temperature oscillation incubator (HZQ-F160, Beijing donglian har lnstrument manufacture co.,ltd). Unless otherwise indicated, standard reactions were performed by incubating purified enzyme (final concentration 12 μg ml^{-1}) with 0.1 mM CPs (dissolved in acetone), 0.2 mM NADPH (nicotinamide adenine dinucleotide phosphate) in 50 mM sodium phosphate buffer (pH 7.5) and 0.005 mM FAD (Flavin adenine dinucleotide) at 25 °C and 0 °C (immersed in ice water) with mild shaking. After the reaction, samples were quickly moved to 100 °C hot water to deactivate the enzyme. All the removals were performed for three times and statistical significance was determined by one-way analysis of variance (ANOVA) followed by Dunnett's test [12].

2.2.3. Cofactor requirement for hydroxylase activity and CPs removal rate

2,4-Dichlorophenol hydroxylase is bright yellow and its visible absorption spectrum is typical of a flavoprotein. The prosthetic group is FAD since FAD alone reconstituted active enzyme from apoenzyme [28]. The FAD requirement experiment for hydroxylase activity and CPs removal rate at 25 °C and 0 °C were conducted as that of the enzymatic CPs removal method described above. Experiments without addition of FAD were used as control.

2.2.4. Enzyme assay and characterization

The enzyme assay for CPs during removal were determined by monitoring the decrease in absorbance at 340 nm (ϵ_{340} = 6220 M^{-1} cm^{-1}) following the substrate-dependent oxidation of NADPH. Unless otherwise indicated, standard enzyme activity assays were performed by incubating the purified enzyme with 0.1 mM CPs, 0.005 mM FAD and 0.2 mM NADPH in 50 mM sodium phosphate buffer (pH 7.5) at 25 °C or at 0 °C in 500 μl eppendorf tube. The total volume of the reaction mixture is 200 μl. One unit of activity was defined as the amount of enzyme required to consume 1 μmol NADPH per min at 25 °C. Protein concentrations were determined by the BCA method (Novagen® BCA Protein Assay Kit) using bovine serum albumin as the standard. CPs removal rate were measured after 60 min reaction by using UV spectrometry. The removal rate of CPs was calculated by dividing the concentration of the amount of reduction of NADPH by the amount of the initial NADPH.

2.2.5. HPLC analysis

CPs removal rates were also analyzed using HPLC. The detection of CPs reduction using HPLC (not shown) allows discrimination of the true substrate, which elicits NADPH oxidation affection on the results since it had been reported that some substrate analogues evoke the oxidation of NADPH and oxygen consumption without themselves undergoing hydroxylation. HPLC apparatus consisted of a WATERS HPLC 600s controller & 616 pump, and a WATERS 2487 Dual λ Absorbance Detector (Milford, MA, USA). After filtering the samples through 0.22-μM PTFE filters from (Navigator Lab Instrument Co. Ltd., Tianjin), aliquots of 10 μl were then analyzed by HPLC. The data were acquired and processed by means of Empower