RESEARCH ARTICLE

Identification of the metal center of chlorothalonil hydrolytic dehalogenase and enhancement of catalytic efficiency by directed evolution

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Abstract: Chlorothalonil hydrolytic dehalogenase (Chd) is one of two reported hydrolytic dehalogenases for halogenated aromatics, and its catalysis is independent of coenzyme A and ATP. Earlier studies have established that the catalytic activity of Chd requires zinc ions. In this study, the metal center of Chd was systematically investigated. The metal content of Chd was determined by inductively coupled plasma-atomic emission spectrometry (ICP-AES), and there were 2.14 equivalents of zinc/mol of protein, indicating that Chd contains a binuclear (Zn^{2+}-Zn^{2+}) center. It was found that other divalent cations, such as cobalt (Co^{2+}) and cadmium (Cd^{2+}), could substitute zinc (Zn^{2+}) leading to relative activities of 91.6% and 120.0%, whereas manganese (Mn^{2+}) and calcium (Ca^{2+}) could substitute Zn^{2+} leading to relative activities of 29.1% and 57.0%, respectively. The enzymatic properties of these different metal ion-substituted Chd variants were also compared. Error-prone PCR and DNA shuffling methods were applied to directly evolve Chd to generate variants with higher catalytic efficiencies of chlorothalonil. Enhanced Chd variants were selected based on the formation of clear haloes on Luria-Bertani plates supplemented with chlorothalonil. One variant, Q146R/N168Y/S303G, exhibited a 4.43-fold increase in catalytic efficiency, showing the potential for application in the dehalogenation and detoxification of chlorothalonil contaminated-sites.

Keywords: chlorothalonil hydrolytic dehalogenase, metallo-β-lactamase, binuclear center, metal ion substitution, directed evolution

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Received: September 9, 2015; **Accepted:** January 20, 2016; **Published Online:** April 7, 2016

Citation: Chen H, Wang H, Wang T, *et al*. 2016, Identification of the metal center of chlorothalonil hydrolytic dehalogenase and enhancement of catalytic efficiency by directed evolution. *Applied Environmental Biotechnology*, vol.1(1): 30–37. http://dx.doi.org/10.26789/AEB.2016.01.003.

1. Introduction

Dehalogenases encoded by microorganisms play key roles in the dehalogenation and detoxification of halogenated compounds[1]. The chlorothalonil hydrolytic dehalogenase (Chd) characterized in our laboratory catalyzes a direct hydroxyl-substitution of the 4-chlorine atom of the chlorinated aromatic fungicide chlorothalonil (2,4,5,6-tetrachloroisophthalonitrile), which is the second most widely used as an agricultural fungicide in the United States[2,3]. The catalytic mechanism of Chd is different from dehalogenases for short-chain halogenated aliphatic hydrocarbons and carboxylic acids[4–6] and also

different from the 4-chlorobenzoyl-CoA dehalogenase, whose catalytic function is dependent on coenzyme A (CoA) and ATP[7,8]. To the best of our knowledge, Chd is the first reported dehalogenase belonging to the metallo-β-lactamase superfamily. Our previous studies showed that Chd contains a conserved domain of the metallo-β-lactamase superfamily. Moreover, Chd was completely inhibited by metalloprotease inhibitor 1,10-phenanthroline (1 mM), and the catalytic activity was recovered by the subsequent supplementation of Zn^{2+}. All these data showed that the possession of a divalent cation is essential for Chd activity.

Metallo-β-lactamases requires a metal cofactor to be enzymatically active. *In vivo*, metallo-β-lactamases probably function in the Zn^{2+}-bound form, but activity is also observed in the Cd^{2+}- or Co^{2+}-bound forms of the enzyme[9–11]. Despite the existence of a pattern for binuclear zinc binding, a structure of metallo-β-lactamase containing only a single zinc ion also has been reported[12]. Whether the Zn^{2+} in the catalytic center of Chd can be substituted by other metal ions and whether Chd contains a mono or binuclear center remain unclear.

Directed evolution is usually used to evolve proteins with desirable properties, and it has been reported to successfully generate enzyme variants with a broader substrate range, enhanced catalytic activity, and thermostability[13–18]. Chd can only dechlorinate one of four chlorines from chlorothalonil to generate 4-hydroxy-trichloroisophthalonitrile (4-TPN-OH); therefore, the substrate range and the catalytic activity of Chd still may be improved. The potential utility of Chd would be greatly improved if its substrate range or catalytic activity could be broadened and enhanced. In this paper, the metal center of Chd was systematically investigated, including the metal ion content (binuclear or mononuclear center) and the possibility for metal ion substitutions. Error-prone PCR and DNA shuffling methods were also used to generate Chd variants with enhanced catalytic efficiency. The investigation of the metal center allows us to know much more about the catalytic mechanism of Chd, while the directed evolution of Chd may lead to potential biological solutions for efficient dehalogenation and detoxification of chlorothalonil contaminated-sites.

2. Materials and Methods

2.1 Chemicals

Chlorothalonil (99.3% purity) was purchased from Sigma-Aldrich (St. Louis, MO). 1,10-phenanthroline was purchased from J&K Chemical Ltd (Beijing, China). All other reagents used in this study were of analytical reagent grade. T4 DNA ligase, deoxynucleoside triphosphates (dNTPs), and restriction enzymes were purchased from Takara Biotechnology Co. Ltd. (Dalian, China). PCR primers were synthesized by Invitrogen Biotechnology Co., Ltd. (Shanghai, China).

2.2 Bacterial Strains

The chlorothalonil dechlorinating strain, *Pseudomonas* sp. CTN-3[2], was stored in our lab. *Escherichia coli* DH5α and BL21 (DE3) strains were used for genetic construction and protein expression, respectively.

2.3 Metal Ion Content Determination

The wild-type Chd was expressed and purified as described previously[2]. The purified Chd was first dialyzed for 24 hrs to remove non-binding metals. The dialysate used was 20 mM Tris-HCl (pH 7.9) and was changed several times. Then, Chd was digested with HNO_3. The metal contents were determined by inductively coupled plasma-atomic emission spectrometry (ICP-AES) (Optima 2100DV, Perkin Elmer). Glassware, test tubes, and tips were pretreated by HNO_3 to prevent metal ion contamination. The metal content value reported is an average of readings from two independent experiments.

2.4 Zinc Ions Replacement

The purified wild-type Chd was incubated in 20 mM Tris-HCl buffer (pH 7.9) containing 10 mM 1,10-phenanthroline at 4°C for 24 hrs, and then dialyzed against deionized water (pH 7.0) for 24 hrs to remove 1,10-phenanthroline-Zn^{2+} and excess 1,10-phenanthroline. The apoenzyme of Chd (apo-Chd) prepared in this manner was completely inactive. Incubation of the enzyme without 1,10-phenanthroline was also used as the control. Different divalent metal ions (Co^{2+}, Cd^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+}, Mg^{2+}, Hg^{2+}, Cu^{2+}, Ni^{2+}, Cr^{2+}, and Fe^{2+}) were supplemented to apo-Chd system to a final concentration of 0.5 mM, and then the mixture was dialyzed against deionized water to remove excess metal ions. The relative activities of these metal ion-substituted Chd were assayed to see whether these metal ions restored the catalytic activity.

2.5 Random Mutagenesis

A mutagenesis library was generated by error-prone

PCR with the genomic DNA of strain CTN-3 as the template. The primer pair F1/R1 5'-ATCAGAATCCCCGCCTGGATGTTCCGG-3' (underlined, BamHI site) and 5'-CAATAAGCTTAGGCCTGGCTGCGAGAT-3' (underlined, HindIII site) was used. To obtain the desired level of mutation (2 to 3 nucleotide substitutions per 1 kb of gene), conditions used for error-prone PCR were optimized; the reaction mixture contained 5 mM $MgCl_2$, 0.2 mM $MnCl_2$, 0.2 mM dATP and dGTP, 1 mM dCTP and dTTP, 25 pmol of each oligonucleotide primer, 20 ng of template, and 2 U of *Taq* polymerase. PCR was performed for 30 cycles consisting of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 1 min. Mutagenic PCR products were digested with BamHI and HindIII and were gel purified with a purification kit (Shanghai Genebase Gene-Tech Co., Ltd.). Purified PCR products were ligated with the same enzyme digested pMD-18T to generate pMD-chd. *E. coli* DH5α cells harboring pMD-chd were plated onto Luria-Bertani (LB) agar plates containing 100 μg/ml ampicillin and 50 μg/ml chlorothalonil for prescreening.

2.6 DNA Shuffling

The dechlorinating activity-improved variants from the first-round of random mutagenesis were used as templates for DNA shuffling[15]. The *chd* genes from different variants were equally mixed and then digested with 0.002 U of DNase I (Fermentas MBI) at 37°C for 15 min. The reaction was stopped by heating at 100°C for 10 min. DNA fragments of 100–250 bp were isolated from a 2% agarose gel with a DNA recovery kit (Shanghai Generay Biotech Co., Ltd). Approximately 0.1 g of the fragmented templates was mixed together and reassembled in a 25 μl volume of primerless PCR by using *pfu* DNA polymerase (Promega). Conditions for PCR were as follows: 1 min at 95°C, followed by 60 cycles of 30 s at 94°C, 30 s at 52°C, and 30 s at 72°C, followed by 2 min at 72°C.

The reassembled product was diluted 10 times and used in an additional PCR amplification using the primer pair F1/R1. The 1.0-kb product was double digested with BamHI/HindIII and subcloned to the same enzymes digested pMD-18T to generate a library of Chd variants. The resulting recombinant plasmids were transformed into *E. coli* DH5α for prescreening.

2.7 Prescreening of Chd Variants for Enhanced Hydrolysis of Chlorothalonil

A plate assay based on the formation of clear haloes due to chlorothalonil dechlorination was used for rapid prescreening of enhanced Chd variants. Transformants were plated onto LB agar containing 50 μg/mL chlorothalonil and 100 μg/mL ampicillin and maintained at 15°C for 48 hrs. Colonies with larger clear haloes were screened from those containing the wild-type Chd. These potential colonies were cultured in LB liquid medium and then dropped onto a chlorothalonil plate at the same cell concentrations for a second-round of screening. Colonies with confirmed larger haloes were selected. The *chd* genes in these colonies were double sequenced by Shanghai Invitrogen Biotechnology Co., Ltd, and Chd variants were further expressed and purified for enzymatic assay.

2.8 Expression and Purification of Chd

Plasmid pET29a(+) was used for expression of the Chd variants as an N-terminal fusion to a hexahistidine tag (His6) for easy purification. The *E. coli* BL21 (DE3) cells containing the recombinant Chd were cultivated in LB liquid medium containing 100 μg/mL kanamycin at 37°C with shaking at 200 rpm for approximately 3 hrs. When the OD reached 0.5–0.8 (approximately 6–8 hrs), isopropyl β-D-1-thiogalactopyranoside (IPTG) was added and then the incubation system was shaken at 150 rpm at 30°C. Cells were harvested with continuous centrifugation (6000 ×g for 5 min), then washed and re-suspended in Tris-HCl buffer (20 mM, pH 7.9) at 4ºC and lysed by sonication on ice. All purification procedures were performed at 4ºC. Cell debris and insoluble proteins were removed by centrifugation (12,000 ×g for 10 min at 4ºC). Expressed Chd was purified from the crude extract using Ni-nitrilotriacetic acid affinity chromatography as described previously[2]. The purity of Chd was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was quantified by the Bradford method using bovine serum albumin as the standard.

2.9 Enzyme Kinetics Study

Standard enzyme activity assays were performed in phosphate-buffered saline (PBS, 50 mM, pH 7.0). The enzyme (10 μL) was mixed with 0.2 mM chlorothalonil in 1 mL of PBS, and the reaction mixture was incubated at 37°C for 10 min. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of the metabolite 4-TPN-OH per min from chlorothalonil. The concentration of 4-TPN-OH was measured by HPLC method. Similarly, the kinetic

parameters *k*cat and K_m, optimum pH, pH stability, optimum temperature, and thermostability were determined as described previously[2].

The pH range of Chd and its variants was determined by incubating 10 µL enzyme with 0.2 mM chlorothalonil in 1 mL reaction buffer at 50°C for 30 sec at pH values ranging from 4.0 to 10.0. For pH stability determination, enzyme was pre-incubated at different pH values (ranging from 4.0 to 10.0) at 50ºC for 10 min, and then the remaining activity was assayed as described above. The reaction buffers were Citric acid-NaOH buffer (pH 4.0–5.0), PBS (pH 6.0–8.0), Tris-HCl buffer (pH 9.0), and Glycin-NaOH buffer (pH 10.0), respectively. The optimal temperature was determined analogously by incubating 10 µl enzyme with 0.2 mM chlorothalonil in 1 mL PBS (50 mM, pH 7.0) for 10 min at different temperatures ranging from 4°C to 70°C. To determine the thermo-stability, enzyme was pre-incubated in a water bath at different temperatures (4–70°C) for 1 hr and then the remaining activity was determined.

3 Results

3.1 The Zinc Ion Content of Chd

The native Chd expressed in *E. coli* BL21 (DE3) was purified from the crude extract using Ni-nitrilotriacetic acid affinity chromatography. The SDS-PAGE analysis of the purified Chd yielded a single band with a molecular weight of approximately 36 kDa, showing that Chd was sufficiently purified. The ICP-AES analysis showed that there were 2.283 µg zinc ions in 605.625 µg purified Chd. Because Chd is a monomer, the results showed that the ion content is approximately 2.14 mol of zinc/mol of protein, indicating that Chd has a binuclear center (each Chd molecule contains two zinc ions).

3.2 Substitution of Zinc Ions with Other Metal Ions

The apo-Chd was prepared by the metal-ion chelator 1,10-phenanthroline and was completely inactive. In addition to the Zn^{2+}, the catalytic activity of apo-Chd was fully or partially recovered by the subsequent supplementation of Co^{2+}, Cd^{2+}, Mn^{2+}, or Ca^{2+} (Table 1 and Figure 1). However, subsequent supplementation of Cu^{2+}, Ni^{2+}, Mg^{2+}, Hg^{2+}, Cr^{2+}, or Fe^{2+} could not recover the catalytic activity. It is interesting that the Co^{2+}-substituted Chd had the highest specific activity (20.8±2.9 U/µg) (Figure 1) and catalytic efficiency (1.142 μM^{-1} s^{-1}) (Table 1), indicating that cobalt may also be an excellent metal center for Chd.

Table 1. The kinetic parameters for hydrolysis of chlorothalonil by different metal ion-substituted Chd

Metal ion	k_{cat}/K_m (μM^{-1} s^{-1})	K_m (µM)	k_{cat} (s^{-1})
Zn^{2+}	0.967	154.4±4.2	149.3±3.1
Co^{2+}	1.142	134.4±3.6	153.5±2.9
Cd^{2+}	0.554	157.8±3.9	87.4±2.7
Mn^{2+}	0.375	168.3±2.1	63.1±3.5
Ca^{2+}	0.279	171.3±5.1	47.8±1.1

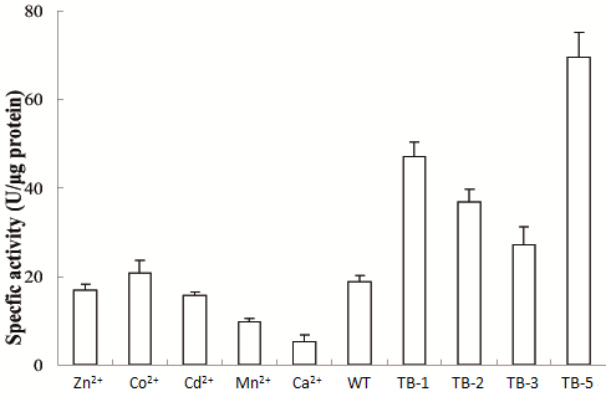

Figure 1. Comparison of the specific activities of different metal ion-substituted Chd and Chd variants. WT, wide type; TB-1, L109H/S303G; TB-2, Q146R/N168Y; TB-3, L243E/E249D; TB-5, Q146R/N168Y/S303G.

3.3 Enzymatic Properties of the Different Metal Ion-substituted Chd

Because the enzymatic activity of Ca^{2+}-substituted Chd was relatively low, only Co^{2+}-, Cd^{2+}-, and Mn^{2+}-substituted Chd were selected for comparison to the Zn^{2+}-substituted Chd. The optimum temperature for catalysis by Zn^{2+}-substituted Chd was found to be 50ºC, which agrees with a previous study[2]. Interestingly, the optimum temperature decreased to 40ºC when Zn^{2+} was replaced by Co^{2+} or Cd^{2+} (Figure 2). The relative activity of Co^{2+}-substituted Chd at 40ºC was about 1.23-fold greater than that of the Zn^{2+}-substituted Chd at its optimum temperature. However, the optimum temperature remained unchanged when Mn^{2+} replaced Zn^{2+}, though the relative activity was only 57.0% of the Zn^{2+}-substituted Chd.

The Zn^{2+}-substituted Chd was relatively stable at 25–50ºC, and it lost less than 20% of its activity when the temperature was increased from 25ºC to 50ºC (Figure 3). However, the Co^{2+}- and Mn^{2+}-substituted Chd were unstable above 40ºC, and the Cd^{2+}-substituted Chd was unstable at 50ºC. The Co^{2+}- and Mn^{2+}-

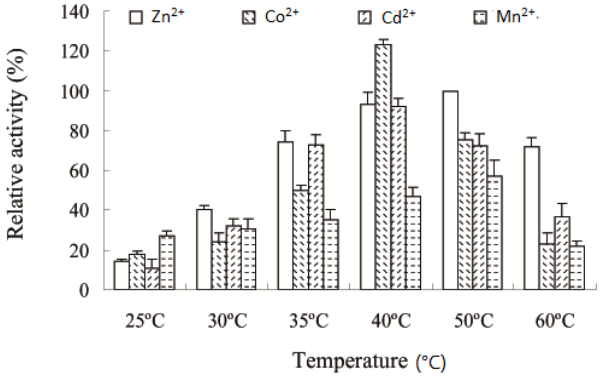

Figure 2. The optimum temperature of different metal ion-substituted Chd. The relative activity of Zn^{2+}-substituted Chd at 50ºC was taken as 100%.

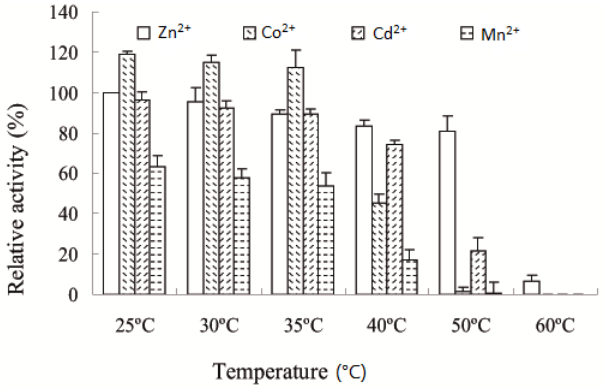

Figure 3. The thermostability of different metal ion-substituted Chd. The relative activity of Zn^{2+}-substituted Chd at 25ºC was taken as 100%.

substituted Chd lost more than 60% of their activities when the temperature was increased from 25ºC to 40ºC, and the Cd^{2+}-substituted Chd lost more than 70% activity when the temperature was increased to 50ºC.

The optimum pH for catalysis was increased from 7 to 8 when Zn^{2+} was substituted by Co^{2+}. The relative activity of Co^{2+}-substituted Chd at pH 8 was about 1.22-fold greater than that of Zn^{2+}-substituted Chd at its optimum pH (Figure 4). The optimum pH remained nearly unchanged when Zn^{2+} was substituted by Cd^{2+} or Mn^{2+}.

All of the metal ion-substituted Chd were fairly stable at pH 6-9 and retained more than 65% of their original activities after pre-incubation at that pH range for 10 min (Figure 5). It seems that metal ion substitution would not significantly change the pH stability.

3.4 Directed Evolution of Chd

Error-prone PCR was first used to evolve Chd. A mutagenic library (about 3,000 clones) was constructed with a mutation rate of between 2 and 3 mutations per

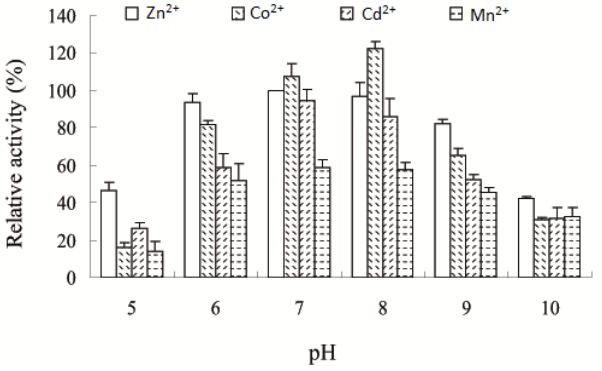

Figure 4. The optimum pH of different metal ion-substituted Chd. The relative activity of Zn^{2+}-substituted Chd at pH 7 was taken as 100%.

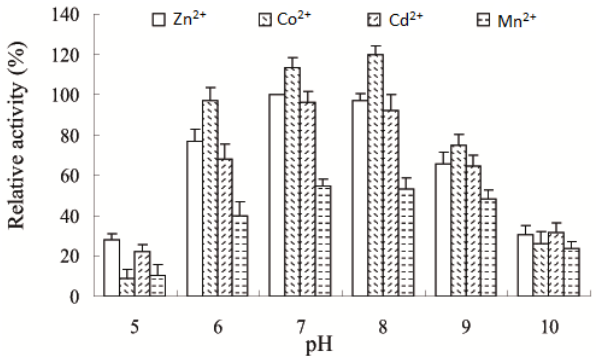

Figure 5. The pH stability of different metal ion-substituted Chd. The relative activity of Zn^{2+}-substituted Chd incubated at pH 7 was taken as 100%.

1,000 bp. Three variants, TB-1 (L109H/S303G), TB-2 (Q146R/N168Y), and TB-3 (L243E/E249D), with large clear haloes as compared to those of the wild-type Chd were screened (Figure 6). It was found that these Chd variants exhibited a 1.44- to 2.51-fold increase in specific activity, and k_{cat} values increased up to 2.95-fold. There was also a modest increase in K_m values (Table 2). The overall improvement in the catalytic efficiency (k_{cat}/K_m) was in line with the improvement in specific activity (Table 2; Figure 1).

The *chd* genes from the above variants were used as templates for further DNA shuffling. One variant, TB-5

Table 2. Kinetic parameters for hydrolysis of chlorothalonil by wild-type Chd and its variants

	k_{cat}/K_m (μM^{-1} s^{-1})	K_m (μM)	k_{cat} (s^{-1})
Wild-type	0.986	153.7±3.8	151.5±4.6
L109H/S303G	2.77	161.1±2.7	446.2±9.4
Q146R/N168Y	2.01	157.9±2.2	317.5±6.2
L243E/E249D	1.29	224.3±4.9	289.3±5.1
Q146R/N168Y/S303G	4.37	126.3±6.8	551.8±11.2

(Q146R/N168Y/S303G), with a large clear halo on the chlorothalonil agar was selected (Figure 6), which is the combination of the mutations from variants L109H/S303G and Q146R/N168Y. The variant Q146R/N168Y/S303G had a 3.70-fold increase in specific activity as compared to the wild-type Chd. The variant had a 3.64-fold increase in k_{cat} value and a modest decrease in K_m with a 4.43-fold increase in catalytic efficiency (4.37 μM^{-1} s^{-1}) (Table 2), indicating that the variant Chd had a very good affinity for the substrate chlorothalonil.

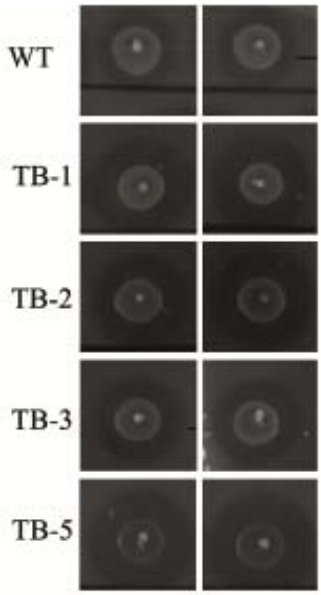

Figure 6. Clear halos formed on a plate supplemented with 50 μg/ml chlorothalonil by wild-type Chd and Chd variants at 48 hrs. Cell concentrations dropped onto the plate were the same. WT, wide type; TB-1, L109H/S303G; TB-2, Q146R/N168Y; TB-3, L243E/E249D; TB-5, Q146R/N168Y/S303G.

4. Discussion

β-Lactamases have been grouped into four classes (A–D) according to sequence homology[19]. Class A, C, and D enzymes use an active site serine residue as a nucleophile, whereas class B lactamases (generically termed metallo-β-lactamases) employ a metal cofactor to be enzymatically active. Zinc clusters have been observed in the active sites of many enzymes[20]. Seven structures that contain at least two zinc ions have been examined: alkaline phosphatase[21], phosphotriesterase[22], Klenow fragment of DNA polymerase I[23], P1 nuclease[24], phospholipase C[25], aminopeptidase[26], and leucine aminopeptidase[27]. However, the enzyme from *Bacillus cereus* functioned reasonably well with only one bound zinc ion[28]. In our study, ICP-AES analysis reached a value of 2.14 equivalents of zinc/mol of protein, showing that Chd contains a binuclear (Zn^{2+}-Zn^{2+}) center. Site-directed mutations of the predicted ligand binding sites of the two zinc ions in Chd resulted in complete loss of catalytic activity, also confirming that each Chd molecule has two zinc ions (data not shown).

It was reported that metallo-β-lactamases can also contain other metal ions for catalytic activity, such as cadmium, cobalt, and manganese. The crystal structure of a Cd^{2+}-bound metallo-β-lactamase from *Bacteroides fragilis* showed that the Cd^{2+}-bound enzyme exhibited the same active-site architecture as that of the Zn^{2+}-bound enzyme, consistent with the fact that both forms were enzymatically active[29]. In our study, the activity of apo-Chd (devoid of Zn^{2+}) could be recovered by subsequent addition of Co^{2+}, Cd^{2+}, Mn^{2+}, or Ca^{2+}, indicating that these divalent metal ions could also act as the metal center. Different divalent metal ion-substituted Chd exhibited different enzymatic properties, such as different optimum temperatures, thermostability, and optimum pH, showing that a different metal ion in the catalytic center might affect enzymatic properties significantly. It was interesting that Co^{2+}-substituted Chd had a higher specific activity than the Zn^{2+}-substituted Chd. However, thermostability results indicated that Zn^{2+} might be the natural metal center of Chd. This investigation of the metal center of Chd allows us to know much more about the catalytic mechanism of the first dehalogenase belonging to the metallo-β-lactamase superfamily. From the results, we can see that Chd also requires a binuclear metal center to be enzymatically active and the natural Zn^{2+} in the catalytic center could also be substituted by other divalent cations, though its enzymatic property was affected.

Directed evolution represents a powerful and simple method to create novel biocatalysts with dramatically improved properties[30]. Error-prone PCR, cassette mutagenesis, and DNA shuffling are commonly utilized to tailor enzymes for specific applications[31]. Because the crystal structure of Chd has not been elaborated and is dissimilar to other proteins, the method of rational design by site-directed mutagenesis based on X-ray crystallography data is not feasible. In this study, we found combination of random mutagenesis and DNA shuffling to be an effective and easy way to evolve Chd. The presence of Q146R, N168Y, and S303G mutations appears to be the most beneficial, as the L109H/S303G, Q146R/N168Y, and Q146R/N168Y/

S303G variants carrying some or all of these mutations exhibited 2.10- to 3.64-fold increases in k_{cat} (Table 2), indicating that these mutations may help to increase the overall hydrolytic rate through elevation of k_{cat}. Variant Q146R/N168Y/S303G, displaying a 4.43-fold increase in catalytic efficiency, also had a modest decrease in K_m, showing an increase in binding affinity. Mutations L243E and E249D in variant L243E/E249D showed a 1.91-fold increase in k_{cat} but also an increase in K_m value (1.46-fold). However, the mechanisms underlying the effect of mutations on catalytic activity are unclear. Unlike the directed mutation of the active-site residues, our directed evolution makes some substitutions that are away from the active sites. Though only a few substitutions were made, the improvement in catalytic efficiency by increasing the hydrolytic rate or improving the affinity with the substrate was obvious. This result indicates that our system functioned effectively in the directed evolution of Chd, which has great potential for the dehalogenation and detoxification of chlorothalonil contaminated-sites.

Author Contributions

Conceived and designed the research: HC SL JJ; performed the experiments: HC HW TW SH; analyzed the data: HC SL JJ; contributed reagents/materials/analysis tools: SL JJ; wrote the paper: HC JJ.

Conflict of Interest and Funding

This work was supported by grants the Outstanding Youth Foundation of Jiangsu Province (BK20130029), the Program for New Century Excellent Talents in University (NCET-12-0892), and the Fundamental Research Funds for the Central Universities (KYZ-201422).

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