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Article

Cloning and Mutational Analysis of Catechol 2,3-dioxygenase from 3,4-Dichloroaniline Degrading Bacterium *Pseudomonas* sp. KB35B

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A gene encoding Catechol 2,3-dioxygenase (CD-2,3) was cloned from 3,4-dichloroaniline degrading bacterium *Pseudomonas* sp. KB35B. Comparison of the deduced amino acids of the CD-2,3 with other CD-2,3 proteins revealed that only three amino acids (Q116, N142 and V215) were particularly different in the CD-2,3 of KB35B. To elucidate how these differences are related with the biochemical properties of the protein, these amino acids were converted into the corresponding residue of other CD-2,3 proteins or Gly, respectively. The effects of amino acid conversion on the catalytic properties of the altered enzymes were then determined, and the results showed that Q116 is a critical amino acid involved in both substrate affinity and catalysis since the conversion of Q116 into other amino acids resulted in the change of both K_m and V_{max}. However, the substitution of N142 showed only change of V_{max}, suggesting that this domain may involve in substrate catalysis. The mutation of V215 domain led to the deficiency of expression, implying that this amino acid is essential for the expression of the CD-2,3.

Key words: *biodegradation, catechol 2,3-dioxygenase, 3,4-dichloroaniline*

The compound 3,4-dichloroaniline (3,4-DCA) is widely used in the production of dyes, drugs and herbicides and it is also a common metabolite in the microbial degradation of various phenylurea, acylanilide and phenylcarbamate herbicides [Lo *et al.*, 1994; Gheewala and Annachhatre, 1997]. 3,4-DCA, however, has been considered potential pollutants due to its toxicity both to invertebrates and vertebrates and to its recalcitrant property [Tixier *et al.*, 2002]. To remove toxic organic compounds such as pesticides, biological and chemical treatments have been suggested [Kim *et al.*, 1997; Kim *et al.*, 2004]. A biological treatment of toxic organic compounds (bioremediation), using microorganisms or enzymes produced from the microorganisms, is often considered an environmentally favorable method [Choi *et al.*, 2005; Choi *et al.*, 2006; Kim *et al.*, 2007; Kim *et al.*, 2008]. To date, however, there have been no unambiguous reports about the bioremediation of soil contaminated by 3,4-DCA.

Recently, *Pseudomonas* sp. KB35B, capable of growth on 3,4-DCA as a sole carbon source, was isolated from sediment [Kim *et al.*, 2007]. The 3,4-DCA degrader strain showed a high level of Catechol 2,3-dioxygenase (CD-2,3) activity in the presence of 3,4-DCA, strongly suggesting that the CD-2,3 is a critical enzyme in the multi-step biodegradation of 3,4-DCA by *Pseudomonas* sp. KB35B [Kim *et al.*, 2007]. Also, it was reported that the conversion of aromatic compounds and chlorine-substituted aromatics to catechol is one of the major metabolic pathways in the bacterial biodegradation [Rodarie and Jouanneau, 2001; Jeong *et al.*, 2003]. However, the properties of CD-2,3 were still remained unknown in 3,4-DCA degrading pathway. In the present paper, we reported the cloning and mutational analysis of the CD-2,3 to determine catalytic properties of the enzyme.

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Abbreviations: CD-2,3, catechol 2,3-dioxygenase; 3,4-DCA, 3,4-dichloroaniline; IPTG, isopropyl β-D-1-thiogalactopyranoside; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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Materials and Methods

Cloning and expression of CD-2,3. In order to clone and express the CD-2,3 gene, *nahH*, of *Pseudomonas* sp. KB35B, it was constructed an expression plasmid (pNahH), which encoded wild type CD-2,3, using polymerase chain reaction (PCR). PCR was carried out using two synthetic oligonucleotides based on the previous report [Kim *et al.*, 2007; accession no. DQ265742]: (sense) 5'-GGAATTCCATATGAATAAAGGTGTAATG CG-3' and (antisense) 5'-ACATCCGGGATCCTTAGGT CATG-3'. PCR was performed at 94°C for 5 min, and then cycled 30 times at 94°C for 1 min, at 55°C for 1 min, at 72°C for 1 min, followed by incubation at 72°C for 5 min. The PCR product was digested by *Nde*I and *Bam*HI restriction enzyme and then ligated into pET21a(+). *Escherichia coli* BL21(DE3) cells harboring pNahH was grown at 37°C in Luria-Bertani medium containing ampicillin (100 μg/mL).

After overnight culture, cells were diluted 50-fold into a fresh medium and grown to OD_{600} of 0.6, at which point the CD-2,3 expression was induced by the addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated at 37°C for 16-20 h. The cells were then harvested by centrifugation, washed in W buffer (100 mM potassium phosphate buffer, pH 7.4), resuspended in the same buffer, and sonicated 3 times at 95 μA for 30 s with an ultrasonicator (Sonic & Materials INC, Newtown, CT). After centrifugation at 20,000 ×g for 10 min at 4°C, the supernatant was taken as crude enzyme and stored at −70°C for later use.

Sequence analysis. Database searches were performed using the BLAST (http://www.ncbi.nlm.nih.gov/BLAST) at the National Center for Biotechnology Information [Altschul *et al.*, 1997]. Multiple sequence alignments were generated using the ClustalW program (http://www.ch.embnet.org/software/ClustalW.html).

Site-directed mutagenesis. Mutants of CD-2,3 were constructed by the two rounds of PCR [Kazuki *et al.*, 2007]. Two external primers and two internal primers (each containing mutated nucleotides) were used to generate PCR products 1 and 2 in separate reaction. The plasmid pNahH containing the *nahH* gene was used as the template. The primers were designed to create an overlap region in the two PCR fragments (Table 1). To reduce the chance of making undesired mutations during the PCR, *EF-Taq* DNA polymerase (Solgent, Daejeon, Korea) was used in this step. The thermal cycling conditions were 95°C for 2 min, 30 cycles of 95°C for 20 s, 62°C for 40 s, and 72°C for 1 min, and 72°C for 5 min. The first round PCR products were then purified by PCR purification kit (Promega, Madison, WI). Each purified products diluted with Tris-EDTA buffer at the rate of 1:30, heated at 95°C for 2 min, and cooled at an ambient temperature. These products were used as the second round of PCR template. The two external primers were used to amplify the full-length mutated *nahH* product. The thermal cycling conditions were equaled to the above PCR conditions. The mutagenesis was further confirmed by sequencing of the PCR products. The final *nahH* products were cut with *Nde*I and *Bam*HI restriction enzymes. The 1.0 kbp fragments containing the full-length *nahH* gene were then ligated into pET21a (+) digested by *Nde*I and *Bam*HI, and transformed into *E. coli* BL21 (DE3) cells. The mutated sequences were confirmed by DNA sequencing. *E. coli* BL21 (DE3) cells harboring mutated *nahH* products were grown and expressed as described above. Protein expression was

Table 1. Oligonucleotide sequences for site-directed mutagenesis of CD-2,3

Substitution	Primers	Sequence
Q116G	Q116G-F	5'-GCCCCCTCTGGGCATGGATTCGAGTTGTAT-3'
	Q116G-R	5'-ATACAACTCGAATCCATGCCCAGAGGGGGC-3'
Q116H	Q116H-F	5'-GCCCCCTCTGGGCATCATTTCGAGTTGTAT-3'
	Q116H-R	5'-ATACAACTCGAAATGATGCCCAGAGGGGGC-3'
N142G	N142G-F	5'-GAGGCTTGGCCGCGCGGTCTGAAAG-3'
	N142G-R	5'-CTTTCAGACCGCGCGGCCAAGCCTC-3'
N142D	N142D-F	5'-GAGGCTTGGCCGCGCGATCTGAAAG-3'
	N142D-R	5'-CTTTCAGATCGCGCGGCCAAGCCTC-3'
V215G	V215G-F	5'-GGCAAGTTCCATCATGGCTCGTTCTTCCTC-3'
	V215G-R	5'-GAGGAAGAACGAGCCATGATGGAACTTGCC-3'
V215A	V215A-F	5'-GGCAAGTTCCATCATGCCTCGTTCTTCCTC-3'
	V215A-R	5'-GAGGAAGAACGAGGCATGATGGAACTTGCC-3'

Mutated nucleotides were underlined.

Table 2. Homology analysis of the CD-2,3 from *Pseudomonas* sp. KB35B

Strain	Protein (length)	Identity (%)	Similarity (%)	Source (accession no.)	E Value[a]
Pseudomonas putida G7	CD-2,3 (307)	98	99	YP_534833	4e-179
Pseudomonas sp. ND6	CD-2,3 (307)	91	94	NP_943120	1e-167
Pseudomonas sp. PD9	CD-2,3 (307)	91	94	ABR24794	1e-167
Pseudomonas sp. ND7	CD-2,3 (307)	91	94	ABR10832	2e-167
P. fluorescens PC20	CD-2,3 (307)	91	94	AAW81680	3e-167
Pseudomonas sp. ND10	CD-2,3 (307)	91	94	ABR10835	4e-167
Pseudomonas sp. ND9	CD-2,3 (307)	91	94	ABR10834	5e-167

Homology search was performed by the BLAST search provided by the National Center for Biotechnology Information (NCBI).

[a]Expect value, which estimates the statistical significance of the match by specifying the number of matches, with a given score, that are expected in a search of a database of this size absolutely by chance.

monitored by SDS-PAGE with Coomassie Brilliant Blue staining.

Enzyme activity and protein concentration. CD-2,3 activity was assayed using the protocol described by Nakanishi *et al.* (1991). The activity of CD-2,3 was measured spectrophotometrically by the increase in absorbance at 375 nm. The reaction mixture contained 1.5 mL of 100 mM potassium phosphate (pH 7.4), 0.1 mL of 10 mM catechol or other substrate, and 1.3 mL of water. The reaction was started at an ambient temperature by adding 0.1 mL of enzyme solution. Absorbance at 375 nm after incubation of 60 s was read, and then the difference of the absorbance was calculated. Protein concentration was determined with bovine serum albumin as the standard.

Steady-state kinetic parameters. The steady-state kinetic parameters of K_m and V_{max} were determined for each mutant. To determine the initial velocity, samples were taken at 60 s after the addition of substrate. Each experimental point is the average value of three times.

Results and Discussion

Analysis of the CD-2,3 from *Pseudomonas* sp. KB35B. Comparison of the deduced primary structure of the CD-2,3 with those of proteins present in the GenBank database indicated that the CD-2,3 showed high amount of similarity with CD-2,3 proteins of *Pseudomonas* sp. (Table 2). Among them, the CD-2,3 of *Pseudomonas putida* G7 [Sota *et al.*, 2006; accession no. YP-534833] showed the highest sequence similarity throughout the entire sequence: 98% identity and 99% similarity (Table 2). The deduced amino acid sequence alignment revealed that only three amino acids (Q116, N142, and V215) were particularly different in the CD-2,3 of KB35B (Fig. 1). From these results, we hypothesized that these amino acids may play an important part in the CD-2,3 of KB35B or not significantly relate to the biochemical properties of the protein. In order to study these issues in more detail, mutational analysis was performed with the point mutated CD-2,3 proteins.

Expression and mutational analysis of wild-type and mutated CD-2,3. A complete *nahH* gene encoding the CD-2,3 from *Pseudomonas* sp. KB35B was cloned and expressed as described in Materials and Methods. *E. coli* BL21 (DE3) cells containing pNahH showed an increased intensity of the band corresponding to about 35 kDa after IPTG induction (lane 2 in Fig. 2). The experimental molecular weight was in correlation with the predicted CD-2,3 protein (about 35 kDa). No enhanced protein band of this size was detectable in the control cells containing the pET21a (+) vector only (lane 1 in Fig. 2). Thus, it was concluded that the 35 kDa band represents the overproduced CD-2,3 protein.

As shown Fig. 1, three amino acids (Q116, N142, and V215) were particularly observed in the CD-2,3 of KB35B. On the contrary, His at 116 position, Asp at 142 position and Ala at 215 position were conserved in all of other CD-2,3 proteins indicating high similarity with the KB35B CD-2,3, respectively. In order to elucidate how these differences are related to the biochemical properties of the protein, the amino acids of the KB35B CD-2,3 (Q116, N142, and V215) were converted into the corresponding residue of the other CD-2,3 proteins or Gly, respectively. Gly may be an influential factor in determining the specific pattern of folding or function of polypeptide chains since this is the smallest amino acid [Neurath, 1943]. The effects of amino acid conversion on the catalytic properties of the altered enzymes were then determined.

Mutants of Q116 and N142 were functionally expressed (Fig. 2). However, the mutation of V215 into other amino acids resulted in the deficiency of protein expression, implying that this amino acid is essential for the

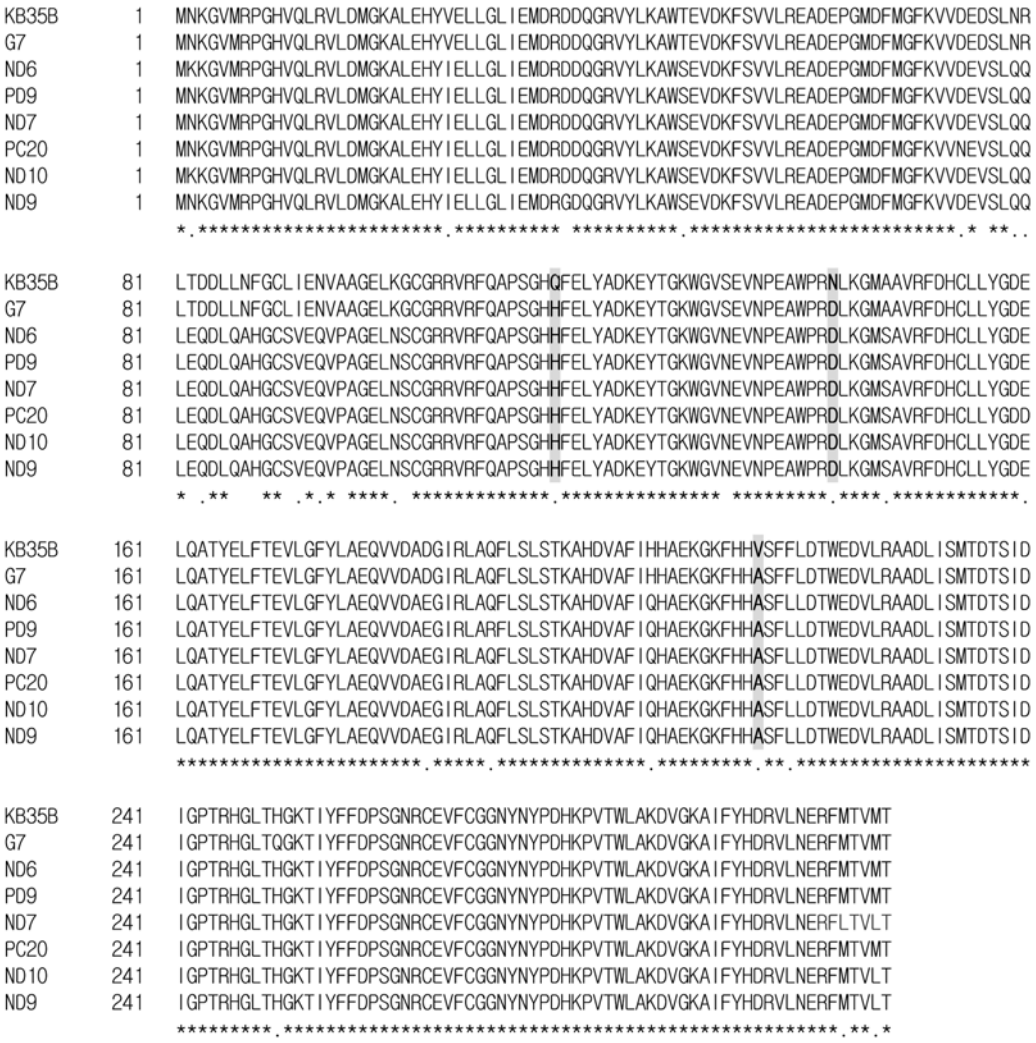

Fig. 1. Sequence alignment of the CD-2,3 from *Pseduomonas* sp. KB35B with other CD-2,3 proteins. An alignment was performed with the ClustalW program. Identical and similar amino acid residues are indicated by asterisks and dots, respectively. The numbers indicate positions of the amino acid residues. *Pseudomonas putida* G7, accession no. YP_534833; *Pseudomonas* sp. ND6, accession no. NP_943120; *Pseudomonas* sp. PD9, accession no. ABR24794; *Pseudomonas* sp. ND7, accession no. ABR10832; *P. fluorescens* PC20, accession no. AAW81680; *Pseudomonas* sp. ND10, accession no. ABR10835; *Pseudomonas* sp. ND9, accession no. ABR10834.

expression of the CD-2,3 (Fig. 2). The specificity of wild-type and mutant CD-2,3 to catechol and its derivatives was shown in Table 3. The wild-type CD-2,3 readily oxidized catechol and 4-methylcatechol. In addition, 3-methylcatechol and 4-chlorocatechol were also substrates under the same conditions. The wild-type CD-2,3, however, did not oxidize 3,5-chlorocatechol, 4,5-chlorocatechol, tetrachlorocatechol, and 3-florocatechol. Although most of mutant proteins showed similar specific activities in general range compared to those of wild -type, some differences were observed. Q116G led to 2-fold increasing of CD-2,3 activity against 4-methylcatechol and 4-chlorocatechol (Table 3). N142 mutants also exhibited different specific activity signatures for all active substrates and catechol, respectively (Table 3). The remarkable differences of substrate specificity in Q116 and N142 mutants reflected a conformational change in the active site that interacts with the corresponding substrate. Therefore, we hypothesized that these amino acids, Q116 and N142, are all essential for correct domain closure and substrate affinity. In order to verify this hypothesis, the steady-state kinetic parameters of wild-type and mutants were determined.

All V215 mutants lacked detectable activity (Table 3) and the expression was also not detected (lane 5 and 6 in Fig. 2). It has been known that Val is usually found in the interior of proteins and is seldom useful in routine biochemical reactions and plays a role in maintaining correct conformation due to its hydrophobic nature. Thus, the substitution of Val in the interior of protein may result

Table 3. Substrate specificity of wild-type and mutated CD-2,3 proteins

Proteins	Relative activity (%)[a]							
	Catechol	3-Methyl catechol	4-Methyl catechol	4-Chloro catechol	3,5-Chloro catechol	4,5-Chloro catechol	Tetrachloro catechol	3-Floro catechol
Wild-type	100±19.70	17±6.15	92±17.90	20±8.60	≤5	≤5	≤5	≤5
Q116G	103±28.27	20±5.28	183±28.08	55±16.05	≤5	≤5	≤5	≤5
Q116H	103±8.69	18±6.11	74±16.65	23±4.38	≤5	≤5	≤5	≤5
N142G	41±9.47	5±1.61	29±5.86	8±0.83	≤5	≤5	≤5	≤5
N142D	160±8.03	24±1.18	90±21.07	31±8.94	≤5	≤5	≤5	≤5
V215G	-[b]	-	-	-	-	-	-	-
V215A	-	-	-	-	-	-	-	-

[a]Relative activity was obtained to compare with that of the wild-type and expressed as the mutant/wild-type ratio.
[b]Not determined. The data is the average of the triplicate experiments.

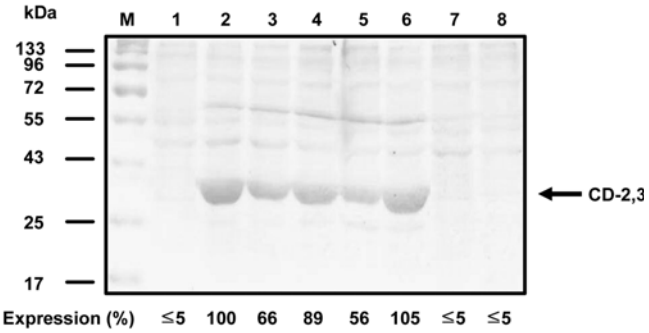

Fig. 2. Expression of wild-type and mutated CD-2,3 proteins. Each of 40 μg crude extract was subjected for SDS-PAGE. CD-2,3 expression was evaluated by densitometry. M, standard protein marker; lane 1, control (vector); lane 2, wild-type; lane 3, Q116G; lane 4, Q116H; lane5, N142G; lane 6, N142D; lane 7, V215G; lane 8, V215A.

in the deficiency of expression due to improper folding. Considering above, we suspected that V215 is a essential amino acid for the expression of the CD-2,3. Also, we speculated that A215 conserved in other CD-2,3 proteins of *Pseudomonas* sp. will be essential for the proper folding in the other CD-2,3 proteins due to its hydrophobic nature such as Val.

Kinetic analysis of CD-2,3 mutants. The kinetic analysis of Q116G mutant revealed that K_m and V_{max} toward all substrates were mostly decreased compared to those of wild-type (Table 4). In particular, it was also observed that more than 50% of the V_{max} against all substrates reduced. However, Q116H mutant led to only change of kinetic parameters toward 4-methylcatechol and 4-chlorocatechol, of which were increased both K_m and V_{max} values, not catechol and 3-methylcatechol, likely due to the less disruptive effect of the Gln to His mutation on protein folding. Our results suggested that Q116 is a critical amino acid in both substrate affinity and catalysis of CD-2,3.

N142G mutation led to only change of V_{max} compared to those of wild-type and no significant difference was observed in K_m, strongly suggested that the substitution of N142 with Gly provides potentially non-optimal interactions due to difference in side-chain length and the stereochemistry of functional groups (Park *et al*., 2008). N142D mutant also showed the significant change of V_{max} toward catechol and 3-methylcatechol, likely due to the disruptive effect in catalysis of substrates. These results supported the idea that N142 domain is a critical amino acid involved in substrate catalysis.

The results presented in this paper suggested that three amino acids (Q116, N142, and V215) play an important part in the CD-2,3 of KB35B. In addition to providing an insight into the properties of the CD-2,3, the role of the

Table 4. Kinetic analysis of the wild-type and mutated CD-2,3 proteins

Proteins	K_m (μM)				V_{max} (μmole/mg protein/min)			
	Catechol	3-Methyl catechol	4-Methyl catechol	4-Chloro catechol	Catechol	3-Methyl catechol	4-Methyl catechol	4-Chloro catechol
Wild-type	0.26±0.04	0.18±0.03	0.11±0.02	0.13±0.01	5.83±0.25	1.05±0.08	4.57±0.39	3.83±0.13
Q116G	0.13±0.05	0.10±0.02	0.14±0.07	0.08±0.01	2.25±0.16	0.43±0.04	2.11±0.17	1.60±0.06
Q116H	0.27±0.02	0.16±0.03	0.25±0.05	0.22±0.01	5.69±0.35	1.16±0.05	6.19±0.17	4.73±0.07
N142G	0.29±0.05	0.14±0.04	0.10±0.02	0.13±0.00	4.96±0.26	0.49±0.07	3.75±0.08	3.10±0.09
N142D	0.27±0.01	0.12±0.00	0.10±0.03	0.08±0.02	10.34±0.27	0.30±0.01	3.46±0.25	2.36±0.19

conserved or particularly different residues between CD-2,3 proteins will be confirmed. To address these issues, these amino acids will be targeted for site-directed mutagenesis and the properties of the altered enzymes have to determine.

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