Thesis for the Degree of Master of Engineering

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

by

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Mutational Analysis and Characterization of Catechol 2,3-Dioxygenase from 3,4-Dichloroaniline Degrading Bacterium *Pseudomonas* sp. KB35B
(3,4-Dichloroaniline을 분해하는 *Pseudomonas* sp. KB35B 균주로부터 유래한 Catechol 2,3-dioxygenase의 특징)

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Abbreviations

DNA bases

A adenosine C cytidine

G guanosine T thymidine

Amino acid

A,	Ala:	alanine
C,	Cys:	cysteine
D,	Asp:	aspartic acid
E,	Glu:	glutamic acid
F,	Phe:	phenylalanine
G,	Gly:	glycine
H,	His:	histidine
I,	Ile:	isoleucine
K,	Lys:	lysine
L,	Leu:	leucine
M,	Met:	methionine
N,	Asn:	asparagine
P,	Pro:	proline
Q,	Gln:	glutamine
R,	Arg:	arginine
S,	Ser:	serine
T,	Thr:	threonine
V,	Val:	valine
W,	Trp:	tryptophane
Y,	Tyr:	tyrosine

3,4-Dichloroaniline을 분해하는 *Pseudomonas* sp. KB35B 균주로부터 유래한 Catechol 2,3-Dioxygenase의 특징

이 윤 경

부 경 대 학 교 대 학 원 식 품 공 학 과

요 약

염화아닐린계(chloroanilines) 화합물들은 오랜 기간 동안 페인트, 농약, 플라스틱, 제약회사 등의 중요한 intermediates로 사용된다. 이 화합물은 분자 내에 염소원자를 가지고 있기 때문에 토양 미생물의 분해에 대한 저항성을 가지며, 토양 부식물질과의 화학적인 결합으로 토양 내에 장시간 잔류하므로 심각한 환경 오염물질로 문제시 되고 있다. 이전 선행연구에서 유기독성 물질로 환경 중에 잔류하여 토양 및 수질 오염을 유발하는 3,4-DCA를 효과적으로 분해 할 수 있는 미생물인 *Pseudomonas* sp. KB35B 균주를 분리하였다. KB35B균은 특이적으로 catechol 2,3-dioxygenase (CD-2,3)의 활성이 3,4-dichloroanilines의 존재하에 크게 증가한 것으로 나타나 선행연구에서는 CD-2,3이 3,4-DCA 분해에 관여하는 중요한 효소군 중 하나로 추측하였다. 따라서 본 연구에서는 3,4-DCA를 효과적으로 분해하는 KB35B 균주의 분해반응 기구를 효소학적 측면에서 조사하기 위해 CD-2,3 유전자를 클로닝하였다. 클로닝 한 염기서열을 분석한 결과 *Pseudomonas putida* G7과 4개의 아미노산 (Q116, N142, V215, H250)만 다른 것으로 나타나 이 4개의 아미노산이 효소의 활성에 어떤 중요한 역할을 하는지를 알아보기 위해 이 아미노산들을 유사성이 가장 높은 *Pseudomonas putida* G7의 CD-2,3에서 보존되어 있는 아미노산과 Gly으로 각각 변형하였다.

Q116과 H250 아미노산을 각각 Gly, His 및 Gly, Gln으로 변형시킨 결과, 효소반응속도 값(K_m, V_{max})이 둘 다 변화하여 이 아미노산들은 효소촉매(catalysis

site)와 기질친화(substrate binfing site)에 관여하는 아미노산으로 추정된다. N142 아미노산을 Gly과 Asn으로 변형시킨 결과 효소반응속도 값(K_m, V_{max}) 중 V_{max} 값의 활성이 변화한 것으로 나타났으며 이는 N142 아미노산이 효소촉매(catalysis site)에 관여하는 것을 의미한다. V215 부위는 Gly, Ala, Leu 으로 변형시켰더니 효소의 활성이 전혀 나타나지 않았고, 이를 SDS-PAGE로 확인한 결과 단백질 발현이 되지 않았다. 이러한 결과는 V215 아미노산은 CD-2,3의 단백질 folding에 중요한 역할을 하는 아미노산인 것을 의미한다. 효소의 비활성값을 보면 특이적으로 Gly으로 유전자 변형한 효소의 비활성값이 wild-type에 비해 2배 이상 감소한 것으로 나타났는데 이는 아미노산 크기가 작은 글리신이 단백질의 고차구조에서 wild-type 아미노산의 빈 공간을 채우지 못해 효소활성의 변화가 크게 나타난 것으로 추정된다. 유전자 변형시킨 효소의 안정성을 확인하기 위해서 효소의 최적 pH와 pH 안정성을 실험한 결과, mutant 단백질들 간의 큰 차이는 관찰되지 않았으며 최적 pH는 7.0-8.0, pH stability는 pH 6.5-9.0에서 약 80% 이상으로 나타나 wild-type과 유사한 것으로 나타났다.

Introduction

The compound 3,4-dichloroaniline (DCA) is widely used in the production of dyes, drugs and herbicides and is also common metabolites of 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, both biological and chemical treatments have been suggested. A biological treatment of the toxic organic compounds (bioremediation), using microorganisms or enzymes produced from the microorganisms, is often considered as an environmentally favorable method. To date, however, there have been no unambiguous reports about the bioremediation of soil contaminated by 3,4-DCA.

It was recently isolated a bacterium strain, *Pseudomonas* sp. KB35B, capable of growth on 3,4-DCA as sole carbon source. It was also shown that catechol 2,3-dioxygenase (CD-2,3) activity was induced by 3,4-DCA exposure in the cells, strongly suggesting that CD-2,3 is a critical enzyme in the multi-step biodegradation of 3,4-DCA by *Pseudomonas* sp. KB35B (Kim et al., 2007). It has been known that the conversion of aromatic compounds and chlorine - substituted aromatics to catechol is one of the major metabolic

pathways in the bacterial biodegradation. It was also previously reported that the CD-2,3 and its flanking enzymes are involved in the biodegradation of 3,4-DCA in *Pseudomonas* sp. KB35B (Kim et al., 2007). However, the properties of CD-2,3 were still remained unknown in 3,4-DCA degrading pathway. Therefore, the characterizations of CD-2,3 protein such as enzyme kinetic analysis, substrate specificity, and catalytic and substrate- binding sites are necessary.

In the present paper, it was reported the cloning and expression of the corresponding gene, *nahH*, in *Escherichia coli*. It was also carried out site-directed mutagenesis studies to elucidate catalytic and substrate- binding sites. The effect of the point mutations on enzyme activity, substrate specificity, and enzyme kinetic have been evaluated in CD-2,3.

Materials & Methods

1. Materials

The bacterial strain KB35B, which is able to grow on plates containing 3,4-DCA as sole carbon source, was previously isolated from a sediment of Yeosu, Jeonnam, Korea (Kim et al., 2007). To identify the isolated strain, culture morphology, biochemical reactions and 16S ribosomal DNA (rDNA) sequences were investigated. Two oligo- nucleotides, based on the report of Dunbar et al. (2000), were used to determine 16S rDNA of the KB35B (Kim et al., 2007).

2. Methods

2.1. Expression and purification of catechol 2,3-dioxygenase

2.1.1. Cloning, expression and purification of catechol 2,3-dioxygenase

In order to clone and express the CD-2,3 of *Pseudomonas* sp. KB35B, it was constructed an expression plasmid, pNahH, which encoded wild type CD-2,3 using PCR. PCR was carried out using

two synthetic oligonucleotides based on the previous report (Kim et al., 2007; the GenBank accession number DQ265742) : (sense) 5′-GGAATTCCATATGAATAAAGGTGTAATGCG-3′ and (antisense) 5′-ACATC CGGGATCCTTAGGTCATG-3′. For one-step purification of the CD-2,3 pNahH-His, which encoded six successive His at the C-terminus of CD-2,3, was also constructed with two oligonucleotides : (sense) 5′-GGAATTCCATATGAATAAA GGTGTAATGCG-3′ and (antisense) 5′-GTCGAGCTTGCGGCCGCGGTCATGACGGTCATG-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-*BamH*I and *Nde*I-*Not*I restriction enzyme and then ligated into pET21a(+), respectively (Novagen, USA). *E. coli* BL21(DE3) cells harboring pNahH and pNaH-His were grown at 37°C in LB medium containing 100 $\mu g ml^{-1}$ of ampicillin.

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 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 sec with an ultrasonicator (Ultrasonic Ltd, England). After centrifugation at 20,000 x g for 10 min at 4°C, the supernatant was taken as crude enzyme and stored at -70°C for later use.

2.1.2. Purification of catechol 2,3-dioxygenase[His- tag] fusion protein

Affinity chromatography was used for the purification of the CD-2,3-[His-tag] fusion protein, containing 6 successive His sequences at the C-terminus. Four milliliters of the crude enzyme from *E. coli* BL21(DE3)/pNaH-His and 1 mL of Ni-NTA (Ni^{2+}-nitrilotriacetic acid) His • bind resin (Novagen, U.S.A.) were mixed by shaking gently at 4°C for 60 min. The mixture was loaded into a column (Φ 1.5 X 6.7 ㎝) and washed twice with 4 mL of W buffer containing 20 mM imidazole. Protein was eluted with 0.5 mL of E buffer (100 mM potassium phosphate buffer containing 250 mM imidazole, pH 7.4). The crude enzymes and each fraction through the purification procedures were collected and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as previously described by Laemmli (1970). Proteins were stained with Coomassie brilliant blue R-250 (CBB; Sigma-Aldrich Co., USA.).

2.1.3. 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 sec was read, and then the difference of the absorbance was calculated. The value of 44,000 was used as the molar extinction coefficient of 2-hydroxymucoinic acid 6-semi-aldehyde, a product of the enzyme reaction (Yoko et al., 1991).

Protein concentration were determined with bovine serum albumin as the standard (Bradford, 1976).

2.1.4 Analysis of sequence alignment

Database searches were performed using the BLAST (http://www.ncbi.nlm.nih.gov/BLAST) at the National Center for Biotechnology Information (Ribeiro et al., 1995).

2.2. Mutational analysis of catechol 2,3- dioxygenase

2.2.1. 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, Korea) was used in this step. The thermal cycling conditions were 95°C for 2 min, and 95°C; 30 cycles of 95°C for 20 sec, 62°C for 40 sec, and 72°C for 1 min; 72°C for 5 min. The first round PCR products were then purified by PCR purification kit (Promega, Germany). Each purified products diluted with TE 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 *BamH* I restriction enzymes. The 1.0 kbp fragments containing the full-length *nahH* gene were then ligated into *Nde* I and *BamH* I digested pET21a(+) (Novagen, USA), 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 at 37°C in a LB medium containing 100 μgml^{-1} of ampicillin. Next procedures were followed as described in Material and Method section 2.1.1. Protein expression was monitored by SDS-PAGE analysis.

Table 1. Primers for site-directed mutagenesis of *nahH*

Substitution	Primers	Sequence
NahH	NahH-F	5′ - GGAATTCCATATGAATAAAGGTGTAATGCG - 3′
	NahH-R	5′ - ACATCCGGGATCCTTAGGTCATG - 3′
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′
N142Q	N142Q-F	5′ - GAGGCTTGGCCGCGCCAACTGAAAG - 3′
	N142Q-R	5′ - CTTTCAGTTGGCGCGGCCAAGCCTC - 3′
V215G	V215G-F	5′ - GGCAAGTTCCATCATGGCTCGTTCTTCCTC - 3′
	V215G-R	5′ - GAGGAAGAACGAGCCATGATGGAACTTGCC - 3′
V215A	V215A-F	5′ - GGCAAGTTCCATCATGCCTCGTTCTTCCTC - 3′
	V215A-R	5′ - GAGGAAGAACGAGGCATGATGGAACTTGCC - 3′
V215L	V215L-F	5′ - GGCAAGTTCCATCATCTCTCGTTCTTCCTC - 3′
	V215L-R	5′ - GAGGAAGAACGAGAGATGATGGAACTTGCC - 3′
H250G	H250G-F	5′ - CACGGCCTGACTGGAGGCAAGACCATTTAT - 3′
	H250G-R	5′ - ATAAATGGTCTTGCCTCCAGTCAGGCCGTG - 3′
H250Q	H250Q-F	5′ - CACGGCCTGACTCAAGGCAAGACCATTTAT - 3′
	H250Q-R	5′ - ATAAATGGTCTTGCCTTGAGTCAGGCCGTG - 3′

2.2.2. Steady-state kinetic parameters

The steady-state kinetic parameters of V_{max} and K_m were determined for each of the mutants. To determine the initial velocity, samples were taken at 60 sec after the addition of substrate. Each experimental point is the average value of three times. To calculate kinetics of the mutants, the data is expressed as the Michaelis- Menton equation (Shaw et al., 1999).

2.3. pH stability of wild-type and mutant catechol 2,3-dioxygenase

The optimum pH was determined by measuring the activity at an ambient temperature over the pH range of 3.0-9.0 using the following buffers (Kalogeris et al., 2006): 100 mM citrate-phosphate buffer (pH 3.0-7.0), 100 mM potassium phosphate buffer (pH 6.5-8.0), and 100 mM Tris-HCl buffer (pH 7.5-9.0)

For the pH stability analysis, both wild-type and mutants were assessed after incubation at 4°C over 24 hours periods, and the remaining activity was measured (Kim et al., 2008).

Results and Discussion

1. Expression and purification of catechol 2,3-dioxygenase gene, *nahH*, from *Pseudomonas* sp. KB35B

1.1. A key enzyme in the multi-step biodegradation of 3,4-DCA

One of the central metabolic routes for the bacterial degradation of aromatic compounds and chlorine - substituted aromatics is the formation of catechol (Rodarie and Jouanneau, 2001; Jeong et al., 2003). It has been also known that a variety of aromatics including xylene, phenol, toluene, and naphthalene can be degraded channeled into this pathway via conversion to catechol, which is then further degraded to *cis, cis* - muconic acid by the catechol 1,2-dioxygenase (CD-1,2; *ortho* -cleavage pathway) or 2-hydroxymuconic semialdehyde by CD-2,3 (*meta*-cleavage pathway). The 3,4-DCA degrader strain, KB35B, interestingly showed high level of CD-2,3 activity by 3,4-DCA exposure (Fig. 1). However, no activity of CD-1,2 was observed (Kim et al., 2007).

This result strongly supports the idea that the CD-2,3 would be a key enzyme in the multi-step biodegradation of 3,4-DCA by

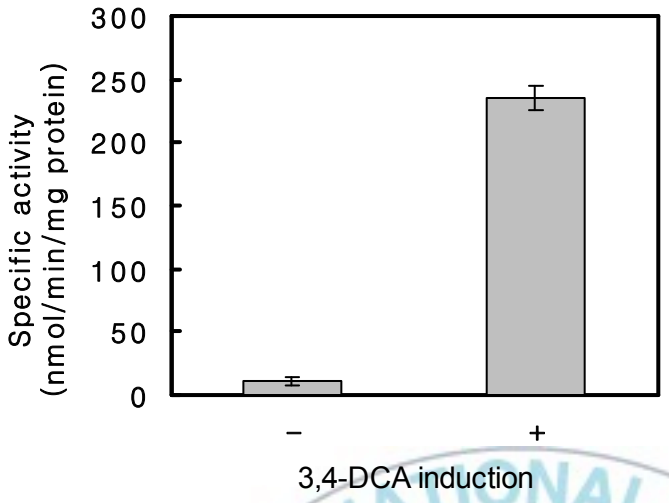

Fig. 1. Catechol 2,3-dioxygenase activity of *Pseudomonas* sp. KB35B. Cells were grown in 1/10 LB for 12 h at 30℃ in the absence (–) or presence (+) of 50 ppm 3,4-dichloroaniline (Kim et al., 2007).

Pseudomonas sp. KB35B. In order to address this issue in more detail, it was tried to clone a related gene involved in the degradation of catechol from *Pseudomonas* sp. KB35B (Kim et al., 2007).

1.2. Cloning of catechol 2,3-dioxygenase gene

A complete gene (*nahH*) encoding the CD-2,3 was cloned from the chromosomal DNA of *Pseudomonas* sp. KB35B as described in Materials and Methods. The nucleotide sequences were shown in Fig. 2. The DNA sequence was translated, and the putative product was compared, using the BLAST algorithm, with all publicly available protein sequences contained in the non-redundant database.

The deduced amino acid sequence from the putative *nahH* gene showed 97, 91, 84, and 83% homologous to *nahH* proteins of *Pseudomonas putida* G7 (YP534833), *Pseudomonas* sp. ND6 (NP863103), *Pseudomonas putida* (NP542866) and, *Pseudomonas putida* MT53 (YP709347), respectively (Table 2).

1.3. Expression and purification of catechol 2,3- dioxygenase

E. coli BL21(DE3) cells containing pNaH-His, which encodes six successive His at C-terminus of NahH, showed an increased intensity of the band corresponding to 36 kDa after IPTG induction.

```
   1 atg/aat/aaa/ggt/gta/atg/cgg/ccc/ggc/cac/gtg/caa/ctg/cgt/gta/ctg/gac/atg/ggc/aag/
       M   N   K   G   V   M   R   P   G   H   V   Q   L   R   V   L   D   M   G   K

  61 gcc/ttg/gag/cac/tac/gtc/gag/ttg/ctt/ggc/ctg/atc/gag/atg/gat/cgt/gac/gac/caa/ggc/
       A   L   E   H   Y   V   E   L   L   G   L   I   E   M   D   R   D   D   Q   G

 121 cgt/gtc/tat/ctg/aag/gcc/tgg/act/gag/gtt/gac/aaa/ttc/tcc/gtg/gtg/ctg/cgc/gaa/gcc/
       R   V   Y   L   K   A   W   T   E   V   D   K   F   S   V   V   L   R   E   A

 181 gat/gag/cca/ggt/atg/gat/ttt/atg/ggt/ttc/aag/gtt/gtc/gac/gaa/gat/agt/cta/aat/cgc/
       D   E   P   G   M   D   F   M   G   F   K   V   V   D   E   D   S   L   N   R

 241 ctc/acg/gat/gat/ctg/ctc/aac/ttt/ggc/tgt/ctg/ata/gaa/aat/gtc/gcc/gcc/gga/gaa/ctc/
       L   T   D   D   L   L   N   F   G   C   L   I   E   N   V   A   A   G   E   L

 301 aaa/ggg/tgt/ggt/cgc/cgc/gtg/cgc/ttc/cag/gcc/ccc/tct/ggg/cat/caa/ttc/gag/ttg/tat/
       K   G   C   G   R   R   V   R   F   Q   A   P   S   G   H   Q   F   E   L   Y

 361 gct/gac/aag/gaa/tac/acg/gga/aaa/tgg/ggg/gtg/agt/gag/gtc/aat/ccc/gag/gct/tgg/ccg/
       A   D   K   E   Y   T   G   K   W   G   V   S   E   V   N   P   E   A   W   P

 421 cgc/aat/ctg/aaa/ggt/atg/gcg/gcg/gtg/cgt/ttt/gat/cat/tgc/ctg/cta/tat/ggt/gac/gaa/
       R   N   L   K   G   M   A   A   V   R   F   D   H   C   L   L   Y   G   D   E

 481 cta/caa/gcc/act/tat/gag/ttg/ttt/acc/gag/gtg/ctc/ggc/ttt/tac/ctg/gcc/gag/caa/gtg/
       L   Q   A   T   Y   E   L   F   T   E   V   L   G   F   Y   L   A   E   Q   V

 541 gtc/gat/gcc/gac/ggg/ata/cgc/ctg/gcc/cag/ttt/cta/agc/ttg/tcg/acc/aag/gcc/cac/gat/
       V   D   A   D   G   I   R   L   A   Q   F   L   S   L   S   T   K   A   H   D

 601 gtg/gct/ttt/atc/cat/cat/gcg/gag/aag/ggc/aag/ttc/cat/cat/gtc/tcg/ttc/ttc/ctc/gat/
       V   A   F   I   H   H   A   E   K   G   K   F   H   H   V   S   F   F   L   D

 661 acc/tgg/gag/gat/gtg/ttg/cgc/gct/gcg/gac/ctg/atc/agc/atg/acg/gac/acc/tcg/atc/gat/
       T   W   E   D   V   L   R   A   A   D   L   I   S   M   T   D   T   S   I   D

 721 atc/ggc/ccg/acc/agg/cac/ggc/ctg/act/cac/ggc/aag/acc/att/tat/ttc/ttc/gac/ccg/tcc/
       I   G   P   T   R   H   G   L   T   H   G   K   T   I   Y   F   F   D   P   S

 781 ggc/aat/cgc/tgc/gag/gtg/ttc/tgc/ggc/ggg/aat/tac/aac/tat/ccg/gat/cat/aag/ccg/gtg/
       G   N   R   C   E   V   F   C   G   G   N   Y   N   Y   P   D   H   K   P   V

 841 act/tgg/ttg/gcc/aag/gat/gtg/ggc/aag/gcg/atc/ttc/tat/cac/gac/cgg/gtg/ctc/aac/gaa/
       T   W   L   A   K   D   V   G   K   A   I   F   Y   H   D   R   V   L   N   E

 901 cga/ttc/atg/acc/gtc/atg/acc/taa
       R   F   M   T   V   M   T
```

Fig. 2. DNA sequences and deduced amino acid sequences of *Pseudomonas* sp. KB35B *nahH* gene. The DNA sequence is numbered on the left.

Table 2. Homology analysis of the catechol 2,3-dioxygenase from *Pseudomonas* sp. KB35B (AY378173)

Strain	Protein	Identity (%)	Similarity (%)	Accession no.
Pseudomonas putida G7 (plasmid NAH7)	catechol 2,3-dioxygenase	97	98	YP534833
Pseudomonas sp. ND6 (pDTG1p50)	catechol 2,3-dioxygenase	91	94	NP863103
Pseudomonas putida (plasmid pWW0)	catechol 2,3-dioxygenase	84	92	NP542866
Pseudomonas putida MT53 (pWW53_74)	catechol 2,3-dioxygenase	83	91	YP709347
Pseudomonas putida MT53 (pWW53_49)	catechol 2,3-dioxygenase	81	90	YP709322
Ralstonia eutropha H16	catechol 2,3-dioxygenase	58	71	YP728708
Thauera sp. MZ1T	catechol 2,3-dioxygenase	57	69	ZP02840994
Azoarcus sp. BH72	catechol 2,3-dioxygenase	56	69	YP933942
Thauera sp. MZ1T	catechol 2,3-dioxygenase	56	69	ZP02841012

The experimental molecular weight was in correlation with the predicted NahH-[His-tag] fusion protein (36 kDa). No enhanced protein band of this size was detectable in the control cells containing the pET21a(+) vector only. Thus, it was concluded that the 36 kDa band represents the overproduced NahH-[His-tag] fusion protein (Fig. 3, lane 1). It was also investigated the effect of a His-tag added to the C-terminus of NahH. No significant difference of activity was observed between wild type NahH and NahH-[His-tag] fusion protein. Thus, the His-tag at the C-terminus did not significantly influence the functional properties of the NahH protein. The purified band had an apparent molecular mass near 36 kDa such as the crude enzyme fraction (Fig. 3, lane 4). Table 3 summarizes the purification of the NahH-[His-tag].

1.4. Characterization of the purified catechol 2,3-dioxygenase

Specificity of the CD-2,3 of *Pseudomonas* sp. KB35B to various catechol and its analogues was shown in Table 4. The purified enzyme is able to oxidize 4-methylcatechol and had a little effect on 3-methylcatechol and 4-chlorocatechol. The CD-2,3 is not oxidized 3,5-dichlorocatechol, 4,5-dichlo- rocatechol, and tetrachlorocatechol. From above results, it was supposed that 3,4-DCA is converted to catechol and further metabolized via *meta*-cleavage pathway.

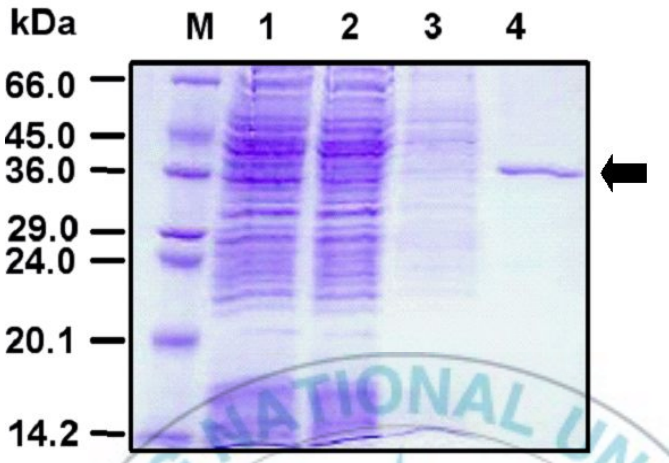

Fig. 3. Expression and purification of catechol 2,3- dioxygenase from *Escherichia coli* BL21 (DE3)/pNahH-His cells. M, standard protein marker; lane 1, crude enzymes (40 μg); lane 2, unbound protein (40 μg); lame 3, wash fraction (5.6 μg); lane 4 ,purified CD-2,3 (0.42 μg).

Table 3. Summary for the purification [His-tag] fusion protein

Fraction	Protein (mg)	Specific activity (μmol · min^{-1} · mg protein^{-1})	Purification rate (fold)
Crude enzyme	3.89	0.71	1
Elute	0.04	9.91	14

The data is the average of the triplicate experiments.

Table 4. Substrate specificity of the purified catechol 2,3-dioxygenase

Substrate	Relative activity (%)[a]
Catechol	100
3-methylcatechol	27.0
4-methylcatechol	60.6
4-chlorocatechol	13.5
3,5-dichlorocatechol	–[b]
4,5-dichlorocatechol	–
Tetrachlorocatechol	–

Assay was performed according to the procedure of Nakanishi et al. (1991).
a, The activity with catechol was defined as 100%.
b, Not determined.

However, it is still unknown how 3,4-DCA is converted to catechol. I supposed to be a gene involved in the conversion of 3,4-DCA to catechol in *Pseudomonas* sp. KB35B. To address these issues, it will be necessary to clone a gene(s) involved in the conversion of 3,4-DCA to catechol.

2. Mutational analysis of catechol 2,3-dioxygenase

It has been known that CD-2,3 is a critical enzyme for the bacterial degradation of aromatic compounds and chrlorine-substituted aromatics. However, the properties of CD-2,3 were still remained unknown. In attempt to elucidate the characteristics of CD-2,3, the functional studies such as enzyme kinetics, substrate specificities, and catalytic and substrate- binding sites are necessary.

As shown in Table 2, The CD-2,3 of KB35B showed the highest amino acidic sequence identity (97%) to the CD-2,3 found in plasmid NAH7 of *P. putida* G7 (Sota et al., 2006; accession no. YP_534833). The analysis of sequence alignment between two proteins revealed that four amino acids are only different at position 116, 142, 215 and 250, implying that these amino acids may play an important part in the CD-2,3 of KB35B (Fig. 4).

In order to characterize of CD-2,3, mutational analyses were performed with the point mutated CD-2,3 proteins.

```
  1 MNKGVMRPGHVQLRVLDMGKALEHYVELLGLIEMDRDDQGRVYLKAWTEVDKFSVVLREA  60
  1 MNKGVMRPGHVQLRVLDMGKALEHYVELLGLIEMDRDDQGRVYLKAWTEVDKFSVVLREA  60
                                                              *
 61 DEPGMDFMGFKVVDEDSLNRLTDDLLNFGCLIENVAAGELKGCGRRVRFQAPSGHQFELY 120
 61 DEPGMDFMGFKVVDEDSLNRLTDDLLNFGCLIENVAAGELKGCGRRVRFQAPSGHHFELY 120
                         *
121 ADKEYTGKWGVSEVNPEAWPRNLKGMAAVRFDHCLLYGDELQATYELFTEVLGFYLAEQV 180
121 ADKEYTGKWGVSEVNPEAWPRDLKGMAAVRFDHCLLYGDELQATYELFTEVLGFYLAEQV 180
                                     *
181 VDADGIRLAQFLSLSTKAHDVAFIHHAEKGKFHHVSFFLDTWEDVLRAADLISMTDTSID 240
181 VDADGIRLAQFLSLSTKAHDVAFIHHAEKGKFHHASFFLDTWEDVLRAADLISMTDTSID 240
               *
241 IGPTRHGLTHGKTIYFFDPSGNRCEVFCGGNYNYPDHKPVTWLAKDVGKAIFYHDRVLNE 300
241 IGPTRHGLTQGKTIYFFDPSGNRCEVFCGGNYNYPDHKPVTWLAKDVGKAIFYHDRVLNE 300

301 RFMTVMT
301 RFMTVMT
```

Fig. 4. Comparison of amino acid sequences of catechol 2,3-dioxygenase of *Pseudomonas* sp. KB35B and *Pseudomonas putida* G7.

The point mutations at Q116, N142, V215, and H250 were carried out by site-directed mutagenesis as described Materials and Methods. Each of amino acid was converted into the corresponding residues of the CD-2,3 found in plasmid NAH7 of *P. putida* G7 or Gly. Gly may be a influential factor in determining the specific pattern of folding of polypeptide chains since this is the smallest residue. Thus, it is of interest to examine certain experimental and theoretical aspects of this hypothesis in relation to the problem of protein structure (Neurath, 1943). As shown in Table 5, protein amounts of wild-type and mutants were similar but the activities of mutants are significantly changed compared to that of the wild-type. The difference of activity may be originated from the difference of CD-2,3 expression between wild and mutant proteins. Therefore, the CD-2,3 expression of all proteins was monitored by SDS-PAGE (Fig. 5). Considering the difference of expression, the relative activity against substrates for wild-type and each mutant in the forward reaction was investigated. The relative activity obtained for mutants was compared to that of the wild-type, NahH, and expressed as the mutant/wild-NahH ratio (Table 6). The activity toward catechol was defined as 100%. However, many of mutants altered at Q116, N142, V215 and H250 are exhibiting different activity against substrates considering the expression levels, suggesting that the amino acids are all essential for correct domain closure and substrate affinity (Table 6). In order to test this hypothesis, it was determined the steady-state kinetic parameters of wild-type and mutants.

Table 5. Specific activity of wild-type and mutated catechol 2,3-dioxygenase

Mutants	Total Protein (mg)	Specific activity against substrates(μmole/mg protein/min)							
		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	0.900	14.0±0.06	2.3±0.01	12.9±0.01	2.8±0.00	0.0±0.00	0.4±0.00	0.1±0.00	0.0±0.00
Q116G	0.817	6.4±0.02	1.3±0.00	11.3±0.04	3.4±0.01	0.0±0.00	0.5±0.00	0.1±0.00	0.1±0.00
Q116H	0.934	14.6±0.02	2.5±0.01	10.5±0.03	3.3±0.01	0.0±0.00	0.3±0.00	0.1±0.00	0.1±0.00
N142D	0.859	31.1±0.02	4.6±0.01	17.4±0.05	6.0±0.02	0.0±0.00	1.1±0.00	0.0±0.00	0.0±0.00
N142G	0.833	8.5±0.02	1.0±0.00	5.9±0.01	1.7±0.00	0.0±0.00	0.2±0.00	0.0±0.00	0.0±0.00
N142Q	0.798	14.6±0.02	2.2±0.00	8.5±0.01	2.9±0.01	0.0±0.00	0.5±0.00	0.0±0.00	0.0±0.00
V215A	0.735	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00
V215G	0.806	0.4±0.06	0.0±0.05	0.4±0.02	0.1±0.04	0.0±0.00	0.0±0.01	0.0±0.00	0.0±0.00
V215L	0.662	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00
H250G	0.836	7.8±0.02	1.3±0.00	6.3±0.01	2.0±0.00	0.0±0.00	0.3±0.00	0.0±0.00	0.0±0.00
H250Q	0.744	11.9±0.02	1.3±0.00	5.0±0.01	1.4±0.00	0.0±0.00	0.2±0.00	0.0±0.00	0.0±0.00

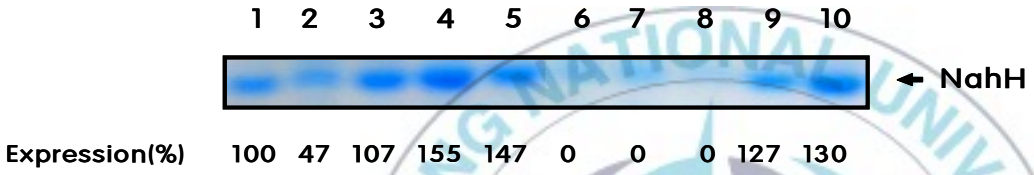

Fig. 5. Expression of wild-type and mutated catechol 2,3-dioxygenase. Each of 10μg crude extract was subjected for SDS-PAGE. lane 1, wild-type (NahH); lane 2, Q116G; lane 3, Q116H; lane4, N142G; lane 5, N142D; lane 6, V215A; lane 7, V215G; lane 8, V215L; lane 9, H250Q; lane10, H250G.

Table 6. Relative activity(%) of wild-type and mutated catechol 2,3-dioxygenase

Mutants	Expression (%)	Relative Activity against substrates(%)							
		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	100	17	92	20	≤5	≤5	≤5	≤5
Q116G	47	103	20	183	55	≤5	9	≤5	≤5
Q116H	107	103	18	74	23	≤5	≤5	≤5	≤5
N142G	155	41	5	29	8	≤5	≤5	≤5	≤5
N142D	147	160	24	90	31	≤5	≤5	≤5	≤5
V215A	0	-	-	-	-	-	-	-	-
V215G	0	-	-	-	-	-	-	-	-
V215L	0	-	-	-	-	-	-	-	-
H250Q	127	72	8	30	8	≤5	≤5	≤5	≤5
H250G	130	45	8	37	12	≤5	≤5	≤5	≤5

2.1. Properties of the Q116

Among 4 sequences examined, Q116 is converted into Gly and His. Q116H shows similar CD-2,3 activity against substrates compared to that of the wild-type. The mutant also shows similar kinetic parameters (K_m and V_{max} values) toward catechol and 3-methylcatechol but not 4-chlorocatechol and 4-methylcatechol, of which are slightly increased both K_m and V_{max} values.

The decrease of CD-2,3 activity against 4-chlorocatechol and 4-methylcatechol at Q116H mutant will be originated from an increase in K_m values (Table 7).

Q116G shows similar CD-2,3 activity against catechol, and 3-methycatechol but not 4-methycatechol and 4-chloro- catechol. The kinetic analysis of Q116G revealed that K_m and V_{max} toward all substrates mostly are decreased compared to that of wild-type. In particular, it was also observed that more than 50% of the V_{max} values reduce. It was supposed that this phenomena may result from the low expression level of Q116G compared to that of wild-type.

From these results, it was surmised that Q116 domain will be a critical amino acid involved in both substrate affinity and catalysis since the mutation of the amino acid at 116 position results in the change of K_m and V_{max} values.

Table 7. Kinetic parameters of the wild-type and Q116 domain

	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

2.2. Properties of the N142

Among 4 sequences examined, N142 is converted into Asn and Gly. N142D shows similar activity toward substrates compared to that of wild-type but catechol (Table 6). N142D activity toward catechol is increased about 1.5-fold compared to wild-type (Table 6). In order to kinetic analysis, the K_m and V_{max} values toward substrates at N142 domain were determined. The K_m and V_{max} values of N142D against substrates are similar to the wild-type but not the V_{max} against catechol, which is increased about 2-fold (Table 8). These results indicate that the mutation at 142 position with Asn affect in catalysis of catechol but not other substrates.

The mutation into Gly at 142 position results in dramatical reduction of CD-2,3 activity against all substrate. However, no significant difference was observed in the K_m and V_{max} values compared to wild-type. These results strongly suggest that substitution of the 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).

Considering above results, it was speculated that N142 domain will be a critical amino acid involved in substrate catalysis since the mutation of N142 with Asn results in the change of V_{max} values.

Table 8. Kinetic parameters of the wild-type and N142 domain

	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
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

2.3. Properties of the V215

Among 4 sequences examined, V215 is converted into Ala, Gly, and Leu. All V215 mutants lack detectable activity (Table 5) and the expression of V215A, V215G, and V215L is also not detected (Fig. 4). 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 their hydrophobic nature. Considering above, it was speculated that V215 domain will be a critical amino acid involved in folding of the CD-2,3 of KB35B.

2.4. Properties of the H250

Among 4 sequences examined, H250 is converted into Gly and Gln. Both mutants at H250 position result in reduction of CD-2,3 activity against all substrate (Table 6). Also, no significant difference was observed in the K_m and V_{max} values of H250G compared to wild-type as like N142G (Table 9). However, H250Q results in the change of the K_m values and dramatical reduction of the V_{max} values, suggesting that H250 domain will be a critical amino acid involved in both substrate affinity and catalysis (Table 9).

Table 9. Kinetic parameters of the wild-type and H250 domain

	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
H250G	0.29±0.07	0.22±0.01	0.16±0.03	0.34±0.00	5.03±0.84	0.75±0.05	4.46±0.18	3.88±0.03
H250Q	0.12±0.04	0.22±0.04	0.24±0.04	0.11±0.04	1.53±0.24	0.77±0.12	3.19±0.11	1.53±0.24

3. pH stability of wild-type and mutant catechol 2,3-dioxygenase

It was investigated the physiological properties of wild-type and mutants. The optical pH for CD-2,3 was determined by incubating the enzyme at different pH values. The enzyme showed optimal activity at a narrow pH range between 7.0 and 8.0 (Fig. 6 - Fig. 12). This is in agreement with mutants. In order to determine pH stability, the protein was incubated for 24 h at 4℃ in each buffer, and the activity was then assayed after the mixture was adjusted to pH 7.4. The wild-type and mutants maintained an activity level in excess of 80% for up to 24 h in a pH range of 6.5-9.0 (Fig. 6 - Fig. 12).

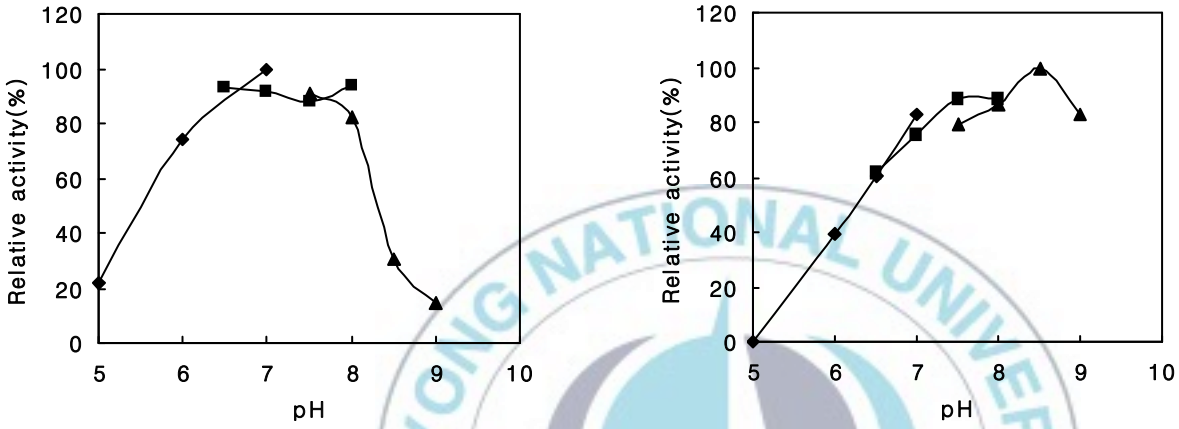

Fig 6. Optical pH (A) and pH stability (B) of wild-type. pH 5.0-7.0, 100 mM citrate-phospate buffer (◆), pH 6.5-8.0 100 mM sodium phosphate buffer (■); pH 7.5-9.0, 100 mM Tris HCl buffer (▲). For pH stability, the protein was incubated at 4℃ for 24 h in each buffer and the activity was then assayed after the mixture was adjusted to pH 7.4.

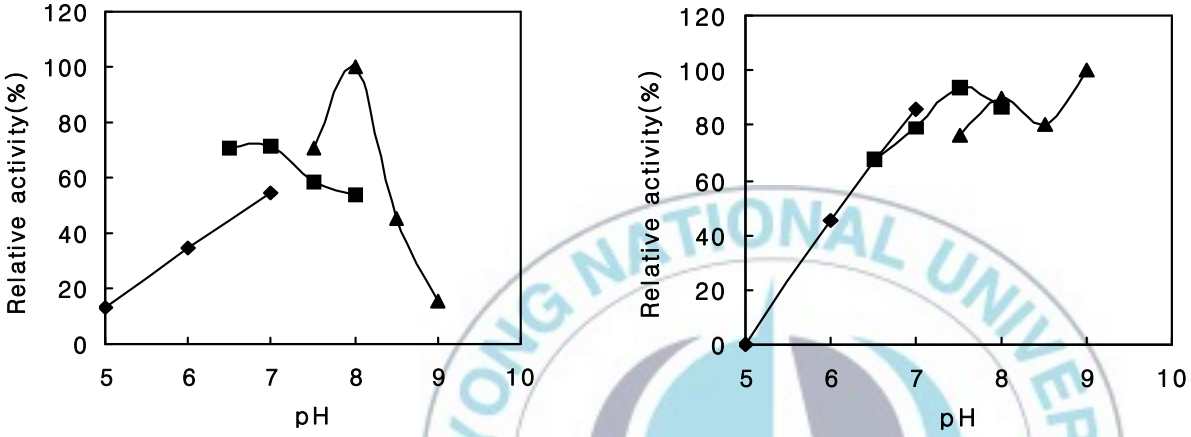

Fig 7. Optical pH (A) and pH stability (B) of Q116G. pH 5.0-7.0, 100 mM citrate-phospate buffer (◆), pH 6.5-8.0 100 mM sodium phosphate buffer (■); pH 7.5-9.0, 100 mM Tris HCl buffer (▲). For pH stability, the protein was incubated at 4℃ for 24 h in each buffer and the activity was then assayed after the mixture was adjusted to pH 7.4.

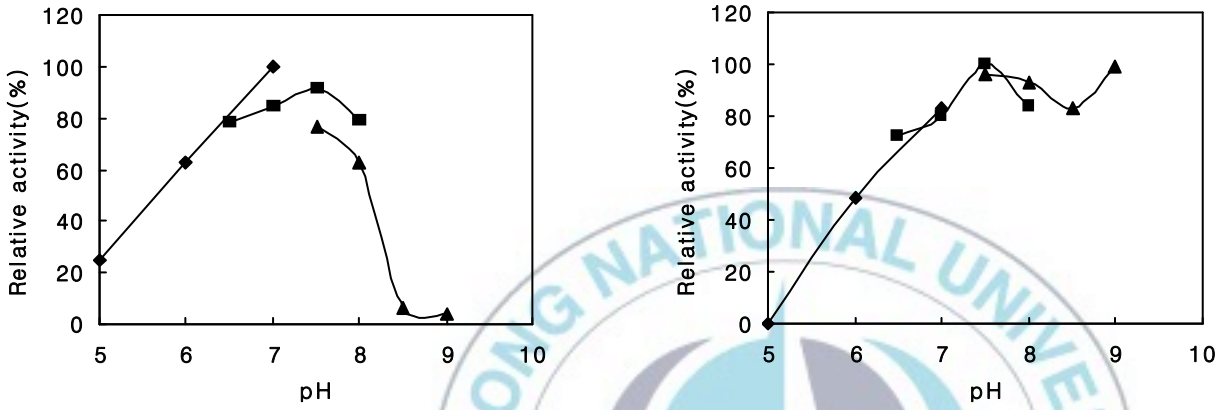

Fig 8. Optical pH (A) and pH stability (B) of Q116H. pH 5.0-7.0, 100 mM citrate-phospate buffer (◆), pH 6.5-8.0 100 mM sodium phosphate buffer (■); pH 7.5-9.0, 100 mM Tris HCl buffer (▲). For pH stability, the protein was incubated at 4℃ for 24 h in each buffer and the activity was then assayed after the mixture was adjusted to pH 7.4.

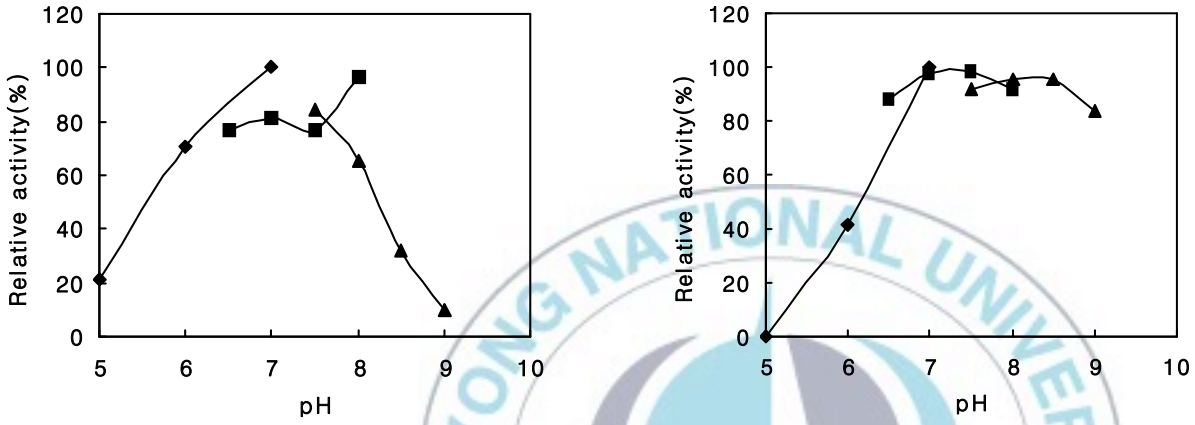

Fig 9. Optical pH (A) and pH stability (B) of N142G. pH 5.0-7.0, 100 mM citrate-phospate buffer (◆), pH 6.5-8.0 100 mM sodium phosphate buffer (■); pH 7.5-9.0, 100 mM Tris HCl buffer (▲). For pH stability, the protein was incubated at 4℃ for 24 h in each buffer and the activity was then assayed after the mixture was adjusted to pH 7.4.

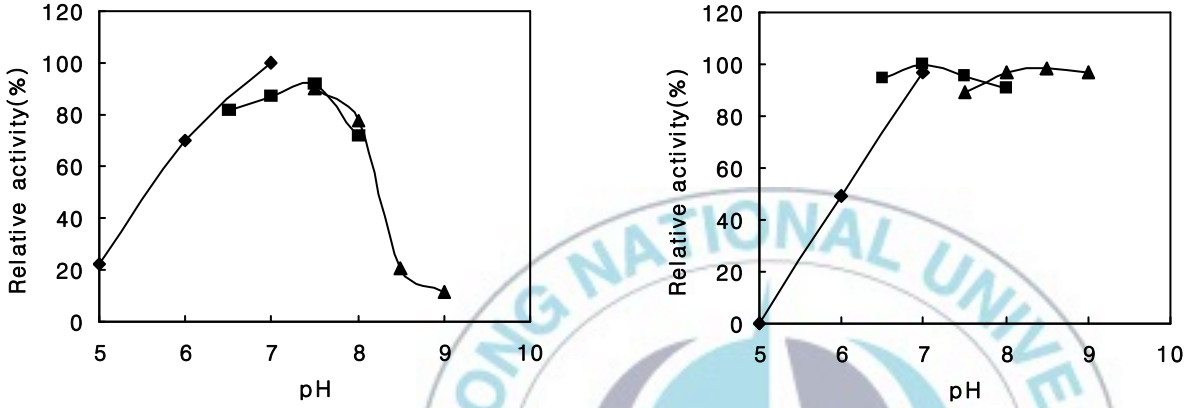

Fig 10. Optical pH (A) and pH stability (B) of N142D. pH 5.0-7.0, 100 mM citrate-phospate buffer (◆), pH 6.5-8.0 100 mM sodium phosphate buffer (■); pH 7.5-9.0, 100 mM Tris HCl buffer (▲). For pH stability, the protein was incubated at 4℃ for 24 h in each buffer and the activity was then assayed after the mixture was adjusted to pH 7.4.

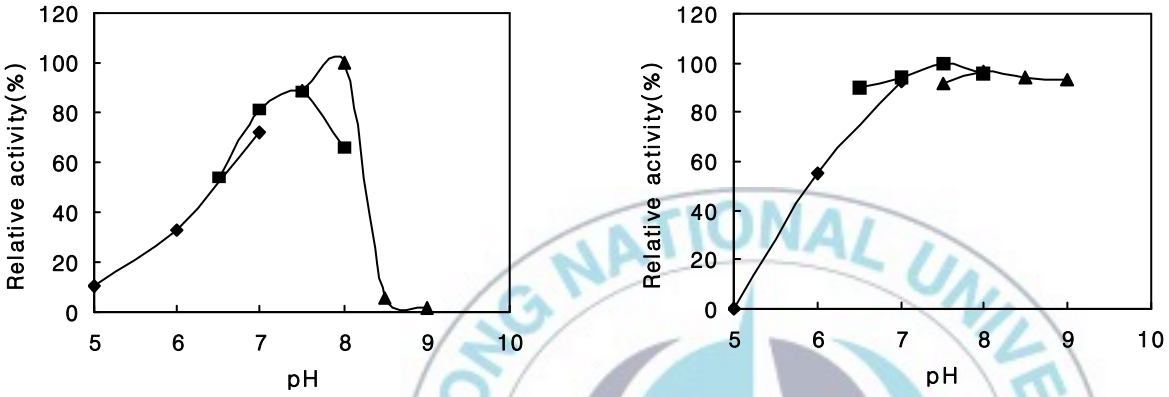

Fig 11. Optical pH (A) and pH stability (B) of H250G. pH 5.0-7.0, 100 mM citrate-phospate buffer (◆), pH 6.5-8.0 100 mM sodium phosphate buffer (■); pH 7.5-9.0, 100 mM Tris HCl buffer (▲). For pH stability, the protein was incubated at 4℃ for 24 h in each buffer and the activity was then assayed after the mixture was adjusted to pH 7.4.

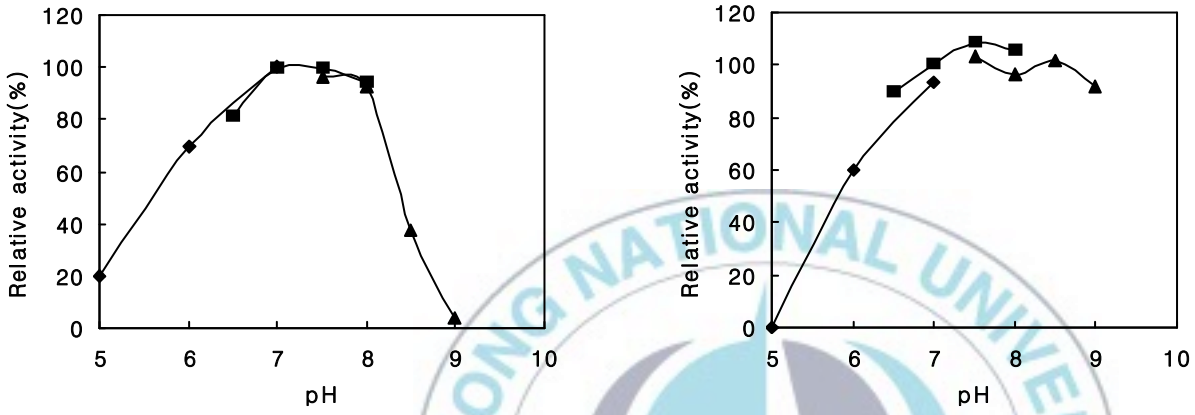

Fig 12. Optical pH (A) and pH stability (B) of H250Q. pH 5.0-7.0, 100 mM citrate-phospate buffer (◆), pH 6.5-8.0 100 mM sodium phosphate buffer (■); pH 7.5-9.0, 100 mM Tris HCl buffer (▲). For pH stability, the protein was incubated at 4℃ for 24 h in each buffer and the activity was then assayed after the mixture was adjusted to pH 7.4.

Conclusion

3,4-Dichloroaniline (DCA) has been considered a potential pollutant due to its toxicity and its high production rate. It previously reported the isolation of *Pseudomonas* sp. KB35B, capable of growth on 3,4-dichloroaniline (DCA) as a sole carbon source. This strain is also able to degrade several chloroanilines and shows a high level of catechol 2,3-dioxygenase (CD-2,3) activity by 3,4-DCA exposure. However, the properties of CD-2,3 were still remained unknown. In attempt to elucidate the characteristics of CD-2,3, the functional studies such as enzyme kinetics, substrate specificities, and catalytic and substrate-binding sites are necessary.

In attempt to elucidate the relation between degradation of 3,4-DCA and CD-2,3 activity, a gene, *nahH*, encoding CD-2,3 was cloned and its properties was characterized. When the amino acid sequences of CD-2,3 were compared to other homologous protein, the CD-2,3 of KB35B was 97% identical to that of *Pseudomonas putida* G7. The analysis of sequence alignment between two proteins revealed that four amino acids is only different at position 116, 142, 215 and 250, implying that these amino acids may play an important part in the CD-2,3 of KB35B. In order to characterize CD-2,3, mutational analyses were performed with the point mutated CD-2,3 proteins. The point mutations at Q116, N142, V215, and H250 were carried out by site-directed mutagenesis. Each of amino acid was converted into the

corresponding residues of the CD-2,3 found in plasmid NAH7 of *P. putida* G7 or Gly.

As a result. it was surmised that Q116 and H250 domain will be a critical amino acid involved in both substrate affinity and catalysis due to the change of K_m and V_{max} values. And the substitution of N142 with Asn results in the change of V_{max} values, suggesting that N142 domain will be a critical amino acid involved in substrate catalysis. Also All V215 mutants lack the activity and the expression, implying that this domain may involve in folding of the CD-2,3 of *Pseudomonas* sp. KB35B.

For the physiological properties of wild-type and mutants, it was investigated the pH stability and optimum pH of wild-type and mutants. As a result, the optimum pH range was between 7.0 and 8.0 and the enzymes kept over 80% of its activity up to 24 h in a pH range of 6.5-9.0.

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