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Expression and Characterization of $catA_1$ (catechol 1,2-dioxygenase I_1) of *Acinetobacter lwoffii* K24 in *Escherichia coli*

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Abstract : Catechol 1.2-dioxygenase I_1 (CD I_1) gene of *Acinetobacter lwoffii* K24, $catA_1$ was expressed in *Escherichia coli* and was partially purified by using a MonoQ column. Expressed CD I_1 had the same molecular weight as purified CD I_1 from *A. lwoffii* K24 on SDS-PAGE. Expressed CD I_1 was also identified by Western blotting and peptide sequencing of N-terminal and internal regions. When compared with purified CD I_1 of *A. lwoffii* K24, expressed CD I_1 had similar substrate specificities and the effects of compounds on enzyme activity. N-terminal amino acid sequence of CD I_1 expressed in *E. coli* was the same as that of purified CD I_1, suggesting that CD I_1 may be under the same posttranslational processing in *E. coli* and *A. lwoffii* K24.
Key words : catechol 1.2-dioxygenase, expression, purification

Catechol 1.2-dioxygenases, which catalyze the intradiol oxygenitive cleavage of catechol to produce *cis,cis*-muconate (Harayama and Kok, 1992; Harwood and Parales, 1996), play a central role in the metabolism of aromatic compounds and are ubiquitous in microorganisms. Catechol 1.2-dioxygenases were purified and cloned from several bacteria, which can use different aromatic compounds as substrates, including *Pseudomonas* (Nakai *et al.*, 1988; Nakai *et al.*, 1990; Nakai *et al.*, 1995), *Acinetobacter* (Neidle and Ornston, 1986; Neidle *et al.*, 1988) and *Frateuria* (Aoki *et al.*, 1984).

We purified, cloned and sequenced two *catA* genes (encoding catechol 1.2-dioxygenases I_1 and catechol 1.2-dioxygenases I_2) from aniline-degrading bacteria *Acinetobacter lwoffii* K24 (Kim *et al.*, 1997). Two catechol 1.2-dioxygenases (CD I_1 and CD I_2) had differences in substrate specificities for catechol analogs, physico-chemical characteristics and gene arrangement ($catB_1$-$catC_1$-$catA_1$ and $catB_2$-$catA_2$-$catC_2$). Especially CD I_1 was expressed twice as much as CD I_2 in *A. lwoffii* K24. So we suppose the two enzymes have a different function and regulation mechanism in *A. lwoffii* K24. In this work, we expressed CD I_1 in *Escherichia coli* by IPTG induction, partially purified and characterized in comparison with purified CD I_1 from *A. lwoffii* K24.

Materials and Methods

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Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1. *Acinetobacter lwoffii K24* is a wild type strain capable of using aniline as a sole carbon and nitrogen source (Kim *et al.*, 1997). *A. lwoffii* K24 was re-identified from *Achromobacter gr. D.V.* K24 (Kim *et al.*, 1993). Bacteria were cultured with constant shaking at 27°C according that previously described (Kim *et al.*, 1997). *Escherichia coli* DH5α was used as cloning host. pCD1-1, pCD1, pCD11 and pCD12 are recombinant plasmids carrying *catA*, *catB* and *catC* genes isolated from the *A. lwoffii* K24 chromosome.

Table 1. Bacterial strains and plasmids used

Strain or pasmids	Relative genotypes and phenotypes	Reference of Source
E. coli		
DH5α	F⁻p80, Δ*lacZ* DM15 (Δ*lacZYA-argF*) U169 *ind1 recA1 hsdR17* (r_k^-, m_k^+) *deoR thi-1 supE44 gyrA96 relAl*	
A. lwoffii		
K24	prototroph	Kim *et al.* (1997)
Plasmid		
pUC 118	Ap^r, lac promoter/operator	
pUC 19	Ap^r, lac promoter/operator	
pCD1	*catA*$^+$ *catC*$^+$; pUC118	Kim *et al.* (1997)
pCD11	*catA*$^+$ *catC*$^+$; pUC19	Kim *et al.* (1997)
pCD12	*catA*$^+$ *catB*$^+$ catC$^+$; pUC118	this study
pCD1-1	*catA*$^+$ *catB*$^+$ catC$^+$; pUC118	this study

Manipulation of DNA

Plasmid DNA was prepared by a QIAGEN plasmid kit (Chatsworth, USA) according to the manufacture's protocols. Restriction endonuclease digestion, ligation of DNA and transformation were performed by the methods of Sambrook *et al.* (1989).

Expression and partial purification of CD I_1

E. coli DH5α carrying pCD12 was grown in 100 ml L-broth containing 50 μg/ml ampicillin to an optical density of 0.4 at 600 nm and incubated for overnight with isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM. The culture was harvested and used for purification. Harvested cells were suspended in 2 ml of 50 mM Tris-HCl (pH 8.0) and broken by a sonicator (Sonics & Materials VCX 400, Danbury, USA). The crude extract was centrifuged at 15,000×g for 10 min. Supernatant fraction was loaded on a HR5/5 MonoQ column of FPLC (Pharmarcia, Uppsala, Sweden). Elution was carried out with a linear gradient of NaCl from 0 to 0.5 M at the flow rate of 0.4 ml/min in 40 min. The elute was monitored at 280 nm, and 0.8 ml fractions were collected and used for enzyme assay. Protein concentration was determined by the methods of Bradford (1976). SDS-polyacrylamide gel electrophoresis was performed by the methods of Laemmli (1970).

Enzyme assay

The activity of catechol 1,2-dioxygenase was determined by measuring the amount of *cis,cis*-muconic acid, a product of enzyme reaction using a UV-spectrophotometer (Aoki *et al.*, 1984). One unit of enzyme activity was defined as 1 μmole of *cis,cis*-muconic acid produced per minute at 24℃. Enzyme activity on catechol analogs and the effect of compounds on enzyme were measured according to the previous methods (Aoki *et al.*, 1984; Broderick and O'Halloran, 1991).

Western blotting

Proteins were electrophoretically transferred from SDS-10% PAGE gels onto polyvinyldifluoride (PVDF) membrane. PVDF membrane was probed with mouse anti-CD I_1 serum (at a dilution of 1: 2,000), followed by goat anti-mouse immunoglobulin G conjugated to alkaline phosphatase (Vector laboratories, Burlingame, USA). Enzyme-labeled bands were detected with an alkaline phosphatase substrate kit II of vector laboratories. Mouse anti-CD I_1 serum was prepared from BALB/C mouse (John *et al.*, 1991).

Peptide sequencing

Partial purified MonoQ fraction was further purified by a a gradient of an HR 5/10 proRPC column of FPLC for peptide sequencing. Elution (0.4 ml/min) was performed with a gradient of 5 to 65% acetonitrile in 60 min at 280nm. Purified protein was digested with endopeptidase Glu-C (Boheringer Mannheim, Mannheim, Germany) according to a previous method (Matsudaira, 1993) and the peptides were applied to a μBondapak C18 column (4×300 mm) in HPLC (Dionex DX-300, Sunnyvale, USA). The digested peptides were separated with a 0~60% acetonitrile gradient and each peptide was manually collected by monitoring at 214 nm by modified methods of Kim *et al.* (1992). The flow rate was 0.8 ml/min. N-terminal sequences were determined by an Applied Biosystems 473A protein sequencer (Foster City, USA).

Results and Discussion

Induction and partial purification of CD I_1

We selected pCD1, which has $catA_1$, $catC_1$ (cording muconolactone isomerase) and partial $catB_1$ (cording muconate-lactonizing enzyme), by colony hybridization and subcloned pCD11 from pCD1 (Kim *et al.*, 1997). In this study, we selected another $catA_1$ containing plasmid, pCD1-1 and subcloned pCD12 from pCD1-1. According to restriction mapping, we found pCD12 have additional 1.2 kbp covering the $catB_1$ upstream region (Fig.1). Sequencing of the catB1 upstream region is now in progress. *E. coli* carrying pCD11 and *E. coli* carrying pCD12 had catechol 1,2-dioxygenase activity by induction of IPTG and their specific activities were more than 0.3 U/mg, respectively. But *E. coli* carrying pCD1 had no enzyme activity because the $catA_1$ gene was located at the reverse direction of the lac promoter. Induced catechol 1,2-dioxygenase was purified from *E. coli* carrying pCD12 with a MonoQ column as described in Materials and Methods. Molecular mass of partially purified catechol 1,2-dioxygenase was about 36 kDa on SDS-PAGE, which is equal to CD I_1 purified from *A. lwoffii* K24 (Fig. 2A).

Partially purified $catA_1$ from *A. lwoffii* K24 and *E.*

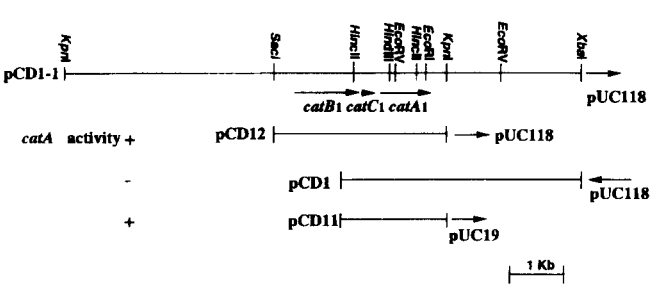

Fig. 1. Restriction maps of the DNA containing cat_1 genes in pCD1-1, pCD1, pCD11 and pCD12. Arrows indicate the direction of transcription. pCD1-1 and pCD1 were selected in colony hybridization. pCD12 and pCD11 were subcloned from pCD1-1 and pCD1, respectively.

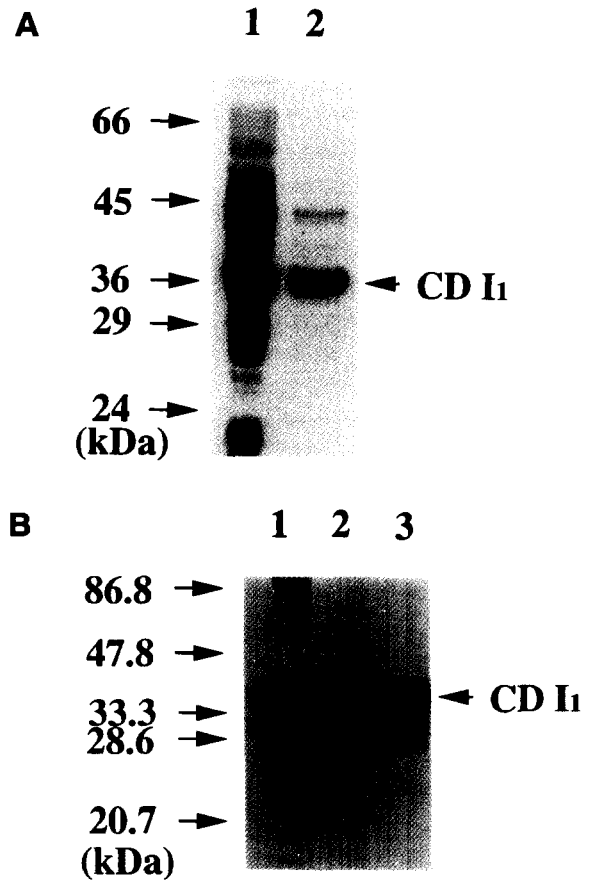

Fig. 2. Expression of CD I_1 in *E. coli*. A. SDS -10% PAGE of partially purified CD I_1. Lane: 1. crude extract of *E. coli* carrying pCD12: 2. MonoQ fraction of *E. coli* carrying pCD12. B. Western blotting of expressed CD I_1. Samples were subjected to SDS-PAGE. electroblotted to PVDF membrane and probed with mouse anti-catechol 1. 2-dioxygenase I_1 serum. Lanes: 1. MonoQ fraction of *A. lwoffii* K24: 2. crude extract of *E. coli* carrying pCD12: 3. MonoQ fraction of *E. coli* carrying pCD12.

coli carrying pCD12 as well as extracts of *E. coli* carrying pCD12 were subjected to Western blot analysis with mouse anti serum against CD I_1 of *A. lwoffii* K24. The same bands of about 36 kDa protein were detected in all of them (Fig. 2B).

Peptide sequencing

The N-terminal amino acid sequence of CD I_1 expressed in *E. coli* was SIKVFGTKEVQDLLKA, which was the same as the N-terminal amino acid sequence of CD I_1 purified from *A. lowffii* K24 (Fig. 3). It was assumed

MAP

	1		5		10
Deduced amino acid [a]	**Met-**	**Ser-Ile-Lys-Val-Phe-Gly-Thr-Lys-Glu-Vla**			
***Acenitobacter lwoffii* K24** [b]		**Ser-Ile-Lys-Val-Phe-Gly-Thr-Lys-Glu-Vla**			
***E.coli* DH5α (pCD12)**		**Ser-Ile-Lys-Val-Phe-Gly-Thr-Lys-Glu-Vla**			

Fig. 3. N-terminal sequences of CD I_1 from *A. lwoffii* K24 and *E. coli*. MAP: [a]methionyl-aminopeptidase. [b]Kim *et al.* (1997).

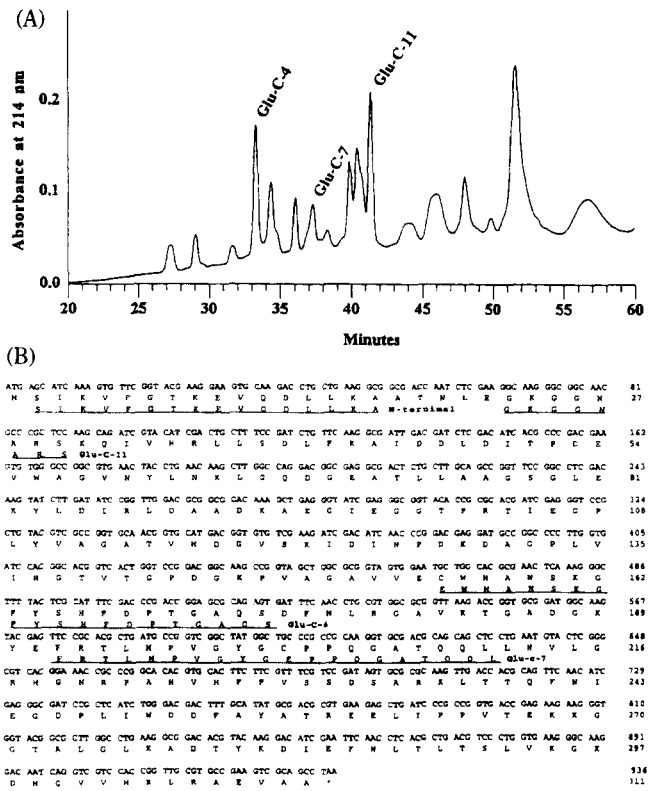

Fig. 4. Peptide sequencing of expressed CD I_1. (A) Chromatogram of Glu-C-digested CD I_1. Purified CD I_1 was digested with endopeptidase Glu-C and separated by μ-Bondapak C18 column (4×300 mm) as described in Material and Methods. Peak fractions was numbered according to the order of elution. Three peaks (Glu-C-4, 7 and 11) were sequenced. (B) DNA and deduced amino acid sequence of CD I_1. The amino acid sequences underlined were N-terminal sequences of enzyme and peptide fragments (Glu-C-4, 7 and 11).

that N-terminal methionine was removed by methionyl-aminopeptidase (MAP). It has been reported that in *E. coli*. most of N-terminal methionine was cleaved by MAP when the proteins had serine as the second amono acid (Hirel *et al.*, 1989: Tobias. 1991). So it is possible that the MAP of *A. lwoffii* K24 has similar substrate specificities and processing mechanism as that of *E. coli*. For sequence analysis of the internal region, protein was digested with endopeptidase Glu-C and digested peptides fragments were prepared (Fig. 4A). Direct amino acid sequencing of three peptide fragments (Glu-4. 7 and 11) showed that they were completely matched with deduced amino acid sequences except for cystein (Fig. 4B). The reason for the difference is that carboxamidomethylated PTH-Cys was coeluted with PTH-Glu and was misreaded as PTH-Glu (Kim *et al.*, 1997).

Substrate specificities of catechol analogues and effects of various compounds on expressed CD I_1

Intradiol cleavage activity of CD I_1 expressed in *E.*

Table 2. Substrate specificities of the expressed catechol 1,2-dioxygenase I_1 to catechol analogues

Substrates	Relative activity (%)	
	Expressed CD I_1	Purified CD I_1[a]
Catechol	100	100
3-Methlcatechol	2.5	2.8
4-Methylcatechol	32.8	39.1
4-chlorocatechol	4.9	5.5
3-Methoxycatechol	4.7	9.9
4-Nitrocatechol	0	0

[a]Kim *et al.* (1997)

Table 3. Effects of various compounds on the expressed catechol 1,2-dioxygenase I_1

Compounds	Concentration (mM)	Relative ctivity (%)	
		Expressed CD I_1	Purified CD I_1[a]
Control		100.0	100.0
Iodoacetic acid	1	111.0	N.T[b]
$HgCl_2$	1	92.5	N.T
NaN_3	1	106.6	101.2
EDTA	1	106.0	99.9
$AgNO_3$	1	21.7	3.0
$FeSO_4$	1	126.4	127.5
$CuSO_4$	1	114.2	121.7

[a]Kim *et al.* (1993)
[b]not tested

coli for catechol and catechol analogues was compared with CD I_1 purified from *A. lwoffii* K24. Expressed CD I_1 showed similar but slightly lower activities than purified CD I_1 (Table 2). The activity of expressed CD I_1 for 3-methoxycatechol was 47.5% of that of purified CD I_1. $AgNO_3$ had a nearly complete inhibition effect (97%) on purified CD I_1 but showed 78.3% inhibition in expressed CD I_1 in *E. coli* (Table 2). Both enzymes were activated by Fe^{2+}.

In conclusion, the *catA*$_1$ gene of *A. lwoffii* K24 was expressed in *E. coli* and characterized. The expressed enzyme (CD I_1) has similar properties with purified CD I_1 from *A. lwoffii* K24.

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