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| 단 보 |

Construction of Overexpression Vectors and Purification of the Oxygenase Component of Alkylphenol Hydroxylase of *Pseudomonas alkylphenolia*

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*Pseudomonas alkylphenolia*의 알킬페놀 산화효소의 과발현 벡터 제작 및 단백질 정제

이 경

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Following construction of expression vectors in *Escherichia coli*, a new procedure involving two-step column purifications with a Fast Performance Liquid Chromatography System was developed for purification of the oxygenase component of alkylphenol hydroxylase of *Pseudomonas alkylphenolia*. From 50 g wet cake of recombinant *E. coli* BL21(DE3)(pJJPMO2) cells, 110 mg of pure protein in a heterodimeric form containing a stoichiometric amount of iron were obtained and it exhibited a specific activity of 147 nmole/min/mg.

Keywords: *Pseudomonas alkylphenolia*, alkylphenol hydroxylase, bacterial multicomponent monooxygenase (BMM), diiron cluster

Pseudomonas alkylphenolia KL28 (KCTC22206) is a Gram-negative bacterium that can utilize alkylphenols with an alkyl group (C_1-C_5) at para or meta position as sole source of carbon and energy (Jeong *et al*., 2003; Lee and Veeranagouda, 2009; Cho *et al*., 2011). A gene cluster consisting of 14 genes, named as *lap*, responsible for the catabolism was previously identified (Jeong *et al*., 2003). The homologies of deduced amino acid sequences and functional studies with subclones showed that *lapKLMNOP* encode alkylphenol hydroxylase (aPH). Substrate specificity studies showed that aPH preferentially oxidizes 3- and 4-alkylphenols to 4-alkylcatechols (Jeong *et al*., 2003). In addition, the deduced amino acid sequences of *lapKLMNOP* exhibit high similarities (43–60%) to those of well-studied *dmpKLMNOP* genes that encode dimethylphenol hydroxylase (dimethyl PH) from *Pseudomonas* sp. CF600 (Shingler *et al*., 1992). The latter enzyme catalyzes hydroxylation of phenol and dimethylphenols to respective catechols and consists of three components, reductase (DmpP), modulator (DmpM), and oxygenase (DmpLMO) (Shingler *et al*., 1992). Reductase (about 42 kDa) contains FAD and 2Fe-2S as cofactors and mediates electron transfer from NADH to oxygenase component. Modulator protein (about 10 kDa) is required for maximal catalytic activity of substrate hydroxylation (Cadieux and Powlowski, 1999). The oxygenase component at a quaternary structure of $(\alpha\beta\gamma)_2$ with a calculated molecular mass of 220 kDa contains a Fe-O-Fe diiron center at the active site and catalyzes hydroxylation reactions in consumption of O_2 and electrons provided by reductase (Cadieux *et al*., 2002). The DmpK has been shown to play a role in assembly of the active form of the oxygenase component of dimethyl PH (Powlowski *et al*., 1997). According to the known biochemical functions of the components of dimethyl PH, homologous functions of the Lap proteins in *P. alkylphenolia* can be envisaged as shown in Fig. 1. Dimethyl PH and aPH belong to a family of BMMs (bacterial multicomponent monooxygenases) which possess characteristic features with a diiron (III) catalytic active site coordinated by four glutamate and two histidine ligands (Sazinsky

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and Lippard, 2006). The BMM members consist of short electron transport components. BMMs play a key role in global methane cycling and biodegradation of toxic compounds (Notomista *et al*., 2003). Thus, the full understanding of BMMs at biochemical levels will aid in solving related global environmental problems. To date, PHs showing activities towards only simple phenols have been purified and characterized from *Pseudomonas* sp. CF600 (Cadieux *et al*., 2002), *Acinetobacter radioresistens* S13 (Divari *et al*., 2003) and *Pseudomonas stutzeri* OX1 (Sazinsky *et al*., 2006). In this study, a method for mass purification of the oxygenase component of aPH from recombinant *E. coli* cells expressing *lap* genes was developed for further studies such as biochemical and crystallographic characterization.

Because the previously constructed aPH expression vector pJJPMO2 is based on a weak promoter pBBR1MCS-5 (Jeong *et al*., 2003), a new vector was constructed for higher expression of aPH as follows. The DNA fragment (854 bp) encoding partial *lapN* and *lapO* was PCR-amplified with primers lapO-HindIII (5′-CACATCAAGAAGCTTGAAGCG-3′, *Hin*dIII underlined) and lapP-XbaI (C5′-TTCGATCTAGAT GGTCAGACA-3′, *Xba*I underlined). The PCR product was cloned into *Hin*dIII/*Xba*I in pK19 (Schafer *et al*., 1994) and the nucleotide sequence was confirmed. The constructed vector was named as pJJO. The rest of aPH genes was retrieved by digestion of pJJPMO2 (Jeong *et al*., 2003) with *Hin*dIII/*Kpn*I and the resulting 3,060-bp fragment was cloned into pJJO at the same restriction sites. A *Kpn*I/*Xba*I (3.9 kb) fragment from the constructed vector (pJJPH1) was again cloned into pTrc99A which contains a strong ptrc promoter (Amann *et al*., 1988) forming pJJPH2 (Fig. 2). The reductase overexpression vector was constructed by cloning the 2.7-kb *Hin*dIII-*Eco*RI DNA fragment of pJJPMO2 into the same sites of pT7-6 (Tabor, 2001) and was named pJJOP2 (Fig. 2). The alkylcatechol dioxygenase (LapB) overexpression vector (pJJC23O2) (Fig. 2) was constructed by cloning of *lapB* gene from the previously constructed pJJC23O (Jeong *et al*., 2003) into *Sal*I/*Sac*I sites of pT7-6. The expression vector (pJJKLM2) of both *lapK* and *lapM* genes was made by deletion of a 2.8-kb *Pst*I fragment in pJJPH2 (Fig. 2). The vectors were transformed into *E. coli* BL21(DE3) and appropriate antibiotic was added for maintaining plasmids in cells.

To make cell-free extracts necessary for sources of enzyme assay, *E. coli* BL21(DE3) strains with plasmids were grown in LB with ampicillin (0.1 mg/ml) overnight at 30℃, and from the seed 1 ml was inoculated into 100 ml LB medium with ampicillin contained in a 500-Erlenmeyer flask. Following 3 h shaking incubation, isopropyl β-D-1-thiogalactopyranoside (IPTG, final concentration of 0.4 mM) and ampicillin were added and cultured for 3 more h. Following cultivation, cells were harvested by centrifugation and 1.4 ml of TG buffer (50 mM Tris-HCl, 5% glycerol, pH 7.0) was added and sonicated for 30 sec. Following centrifugation at 4℃, the cell extracts in 50 μl-aliquots were stored at -72℃ and were used when necessary. For mass cultivation of *E. coli* BL21(DE3)(pJJPH2), NEB Bioflo fermentor (9 L) was used. Cells were cultured in a modified LB medium consisting of NaCl 45 g, yeast extract 90 g, tryptone 90 g, ferrous ammonium sulfate 0.45 g, ampicillin 1.35 g at 30℃, 450 rpm and 4 vvm aeration. Following 2 h fermentation, 1 g IPTG and 1.35 g ampicillin were added again and culture was continued until a turbidity of 6.6 at OD_{600}. After that, cells were harvested by centrifugation and about 100 g of wet cake was obtained. The harvested cells were suspended in 100 ml TG buffer and 50 ml aliquots were stored at -72℃ until use.

Enzyme activity was quantitated using a coupled assay where 4-methylcatechol formed by aPH from substrate *m*-cresol was converted to a yellow-colored ring fission product in the presence of LapB (Cho *et al*., 2009) (Fig. 1). The assay mixture (250 μl) contained in 20 mM Tris-HCl (pH 7.0), *E. coli* BL21(DE3)(pJJC23O2) cell-free extract (alkylcatechol dioxygenase source, 5 μl), *E. coli* BL21(DE3)(pJJOP2) cell-free extract (reductase source, 10 μl), oxygenase source (variable amount) and 2.5 μl

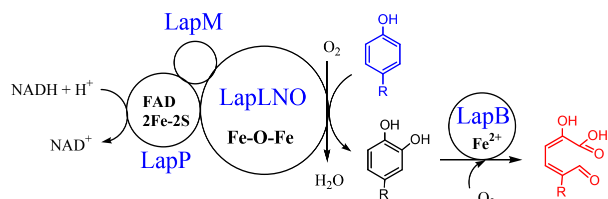

Fig. 1. Diagram showing reactions catalyzed by aPH (LapMPLNO) and alkylcatechol dioxygenase (LapB) yielding a ring-fission product from alkylphenol. The cofactors included in enzymes are also indicated.

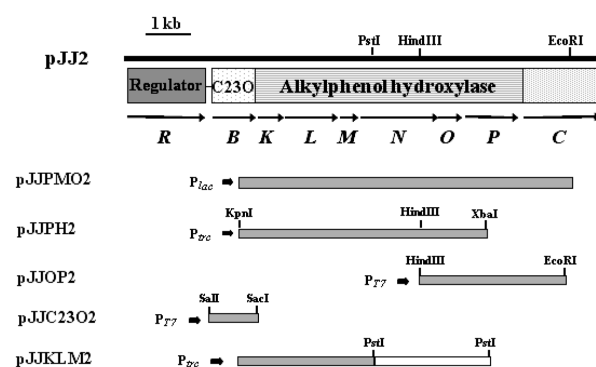

Fig. 2. Genetic maps of a *lap* gene cluster and *lap* gene expression vectors. The promoters controlling the expression of the gene(s) are also indicated. C23O is for alkylcatechol dioxygenase. Plasmids pJJ2 and pJJPMO2 were previously described (Jeong *et al*., 2003). The deleted portion from pJJPH2 is white-boxed in creation of pJJKLM2.

Table 1. Purification steps of the oxygenase component of aPH

	Pooled volume (ml)	Protein concentration (mg/ml)	Total protein (mg)	Activity (U)	Specific activity (mU/mg of LapLNO)[a]
Crude cell extract	120	72	8,640	-[b]	-
Q sepharose	105	7.2	756	81	107
Octyl sepharose	2.1	52.5	110	16.2	147

[a] A unit of aPH (U) was defined as the amount of enzyme that produced 1 μmol product/min under the conditions described. All measurements were made in triplicate.
[b] ND, no detected.

of 0.1 M m-cresol dissolved in methanol. The activity was measured in a kinetic mode of a spectrophotometer (Beckman Co.) by addition of 5 μl of 0.1 M NADH. The increase of absorbance at 420 nm was monitored for 30 sec. The slope was used for calculation of the activity with e_{420nm} = 14 $mM^{-1}cm^{-1}$ (Jeong *et al.*, 2003).

To make a crude cell extract, 50 g of wet cake of *E. coli* BL21(DE3)(pJJPH2) was thawed by addition of 40 ml of TG buffer with 1 μg/ml DNase and was lysed by a Frech pressure as previously described (Lee *et al.*, 1997). The crude cell extract was applied to a Q-Sepharose FF column (GE Healthcare, 5 × 20 cm) previously equilibrated with TG buffer. The column was washed with 200 ml of TG buffer at a flow rate of 2 ml/min and then bound oxygenase was eluted with a 2,100 ml linear KCl gradient (0 – 0.5 M) in TG buffer. Fractions (28 ml) were collected and assayed for aPH activity. Fractions (78-76) were pooled and concentrated to 105 ml over an Amicon YM100 membrane by ultrafiltration. Solid ammonium sulfate (115 g) was slowly added to the solution with stirring. Following centrifugation, the supernatant was applied to a column (2.6 × 26 cm) of Octyl sepharose CL-4B (GE Healthcare) equilibrated with 1 M ammonium sulfate. Oxygenase was eluted with a 450 ml linear gradient of decreasing ammonium sulfate (1.0 – 0.0 M). Fractions (5 ml) were collected and active fractions (113 – 115) were pooled and washed with TG buffer by ultrafiltration. Purified oxygenase was drop frozen in liquid nitrogen in 25 μl aliquots and the protein pellets were stored at -72℃ until use. Protein was purified using a FPLC system (Bio-Rad) at 4℃.

From the purification, 110 mg of oxygenase with a specific activity of 147 nmole/min/mg was obtained from 8,640 mg of cell-free extract proteins (Table 1). The activity of the finally purified oxygenase was independent of the addition of cell-free extract of *E. coli* BL21(DE3)(pJJKLM2), a source of LapK and LapM. The homologous proteins DmpK and DmpM are known to functioning in assembly of active dimethyl PH and in stimulating (more than 10 times) the activity of substrate hydroxylation, respectively (Powlowski *et al.*, 1997; Cadieux and Powlowski, 1999). In addition, DmpM has been reported to be inactive when expressed in *E. coli* (Cadieux and Powlowski, 1999). For these reasons, addition of cell-free extract containing LapK and LapM could have no effects on aPH activity. The specific activity of purified oxygenase is higher compared to that of dimethyl PH which showed a specific activity of about 50 nmole/min/mg in the absence of the modulator component (Cadieux *et al.*, 2002).

The purified oxygenase of aPH exhibited a pale green color due to the presence of diiron clusters at the active site and showed three bands at the expected mass sizes of 58.9, 41.1, and 13.2 kDa in denaturing SDS-PAGE (Fig. 3). No other distinctive contamination bands were observed. The native molecular weight of the protein was estimated to be approximately 220 – 250 kDa from gel permeation chromatography when determined

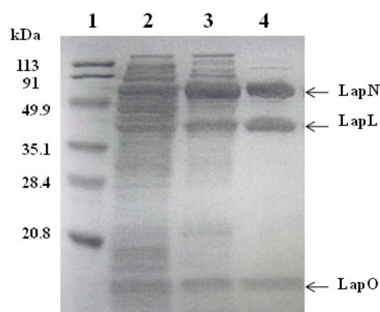

Fig. 3. 12% SDS-PAGE gel showing proteins at each purification step. Lanes: 1, size markers (Bio-Rad); 2, crude cell extract 40 μg; 3, Q-sepharose pool 20 μg; 4, Octyl sepharose pool 10 μg.

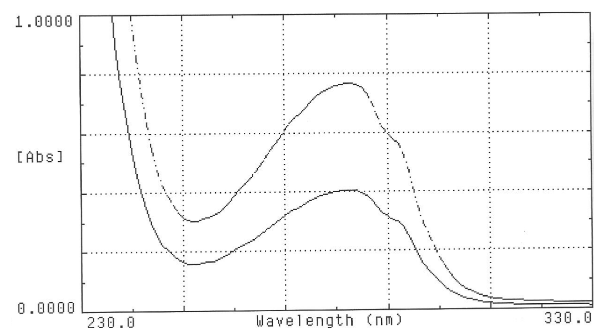

Fig. 4. UV-Vis spectra of the purified oxygenase component of aPH. Upper and lower spectra are with 0.525 and 0.26 mg/ml protein, respectively.

by the method previously described (Haddock and Gibson, 1995). Thus, oxygenase appears to exist as a dimer form, consistent to the crystal structures of BMM PH and toluene/o-xylene monooxygenase hydroxylase from *Pseudomonas stutzeri* OX1 (Sazinsky *et al*., 2004, 2006). The UV-Visible spectrum of purified oxygenase showed a peak at 281 nm (Fig. 4). The calculated e_{281nm} was $1.95 \times 10^5 M^{-1}cm^{-1}$ based on a dimer form. A shoulder was observed at 291 nm, a characteristic of these proteins, indicating a high purity since contamination of other proteins would shield the shoulder absorbance. The estimated iron content was 1.85 mole per αβγ mole as determined by the method previously described (Haddock and Gibson, 1995) and addition of ferrous iron in the assay did not stimulate the activity, indicating almost a stoichiometric amount of iron was bound as a diiron cluster at the active sites of the purified oxygenase. The purified active oxygenase is currently exploited for crystal formation to characterize aPH BMM with unique substrate selectivity and regiospecificity. From the results, aPH can be further modified to increase specific activities and change the substrate specificity towards octyl- and nonylphenols, endocrine disrupting chemicals, and to produce value-added alkylcatechols.

적 요

본 연구에는 대장균에서의 과발현 벡터 개발과 FPLC를 사용한 2단계 컬럼 정제과정을 통해 *Pseudomonas alkylphenolia*의 alkylphenol hydroxylase의 oxygenase 단백질을 다량으로 정제하는 방법을 개발하였다. 재조합 *Escherichia coli* BL21(DE3) (pJJPMO2)의 50 g의 wet cake로부터 110 mg의 heterodimer이며 화학량론적 철을 갖는 순수한 단백질을 정제하였으며 147 nmole/min/mg의 비활성을 보였다.

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References

Amann, E., Ochs, B., and Abel, K.J. 1988. Tightly regulated *tac* promoter vectors useful for the expression of unfused and fused proteins in *Escherichia coli*. *Gene* **69**, 301–315.

Cadieux, E. and Powlowski, J. 1999. Characterization of active and inactive forms of the phenol hydroxylase stimulatory protein DmpM. *Biochemistry* **38**, 10714–10722.

Cadieux, E., Vrajmasu, V., Achim, C., Powlowski, J., and Munck, E. 2002. Biochemical, MÖssbauer, and EPR studies of the diiron cluster of phenol hydroxylase from *Pseudomonas* sp. strain CF 600. *Biochemistry* **41**, 10680–10691.

Cho, J.H., Jung, D.K., Lee, K., and Rhee, S. 2009. Crystal structure and functional analysis of the extradiol dioxygenase LapB from a long-chain alkylphenol degradation pathway in *Pseudomonas*. *J. Biol. Chem.* **284**, 34321–34330.

Cho, A.R., Lim, E.J., Veeranagouda, Y., and Lee, K. 2011. Identification of a *p*-cresol degradation pathway by a GFP-based transposon in *Pseudomonas* and its dominant expression in colonies. *J. Microbiol. Biotechnol.* **21**, 1179–1183.

Divari, S., Valetti, F., Caposio, P., Pessione, E., Cavaletto, M., Griva, E., Gribaudo, G., Gilardi, G., and Giunta, C. 2003. The oxygenase component of phenol hydroxylase from *Acinetobacter radioresistens* S13. *Eur. J. Biochem.* **270**, 2244–2253.

Haddock, J.D. and Gibson, D.T. 1995. Purification and characterization of the oxygenase component of biphenyl 2,3-dioxygenase from *Pseudomonas* sp. strain LB400. *J. Bacteriol.* **177**, 5834–5839.

Jeong, J.J., Kim, J.H., Kim, C.K., Hwang, I., and Lee, K. 2003. 3- and 4-alkylphenol degradation pathway in *Pseudomonas* sp. strain KL28: genetic organization of the *lap* gene cluster and substrate specificities of phenol hydroxylase and catechol 2,3-dioxygenase. *Microbiology* **149**, 3265–3277.

Lee, K., Kauppi, B., Parales, R.E., Gibson, D.T., and Ramaswamy, S. 1997. Purification and crystallization of the oxygenase component of naphthalene dioxygenase in native and selenomethionine-derivatized forms. *Biochem. Biophys. Res. Commun.* **241**, 553–557.

Lee, K. and Veeranagouda, Y. 2009. Ultramicrocells form by reductive division in macroscopic *Pseudomonas* aerial structures. *Environ. Microbiol.* **11**, 1117–1125.

Notomista, E., Lahm, A., Di Donato, A., and Tramontano, A. 2003. Evolution of bacterial and archaeal multicomponent monooxygenases. *J. Mol. Evol.* **56**, 435–445.

Powlowski, J., Sealy, J., Shingler, V., and Cadieux, E. 1997. On the role of DmpK, an auxiliary protein associated with multicomponent phenol hydroxylase from *Pseudomonas* sp. strain CF600. *J. Biol. Chem.* **272**, 945–951.

Sazinsky, M.H., Bard, J., Di Donato, A., and Lippard, S.J. 2004. Crystal structure of the toluene/o-xylene monooxygenase hydroxylase from *Pseudomonas stutzeri* OX1. Insight into the substrate specificity, substrate channeling, and active site tuning of multicomponent monooxygenases. *J. Biol. Chem.* **279**, 30600–30610.

Sazinsky, M.H., Dunten, P.W., McCormick, M.S., DiDonato, A., and Lippard, S.J. 2006. X-ray structure of a hydroxylase-regulatory protein complex from a hydrocarbon-oxidizing multicomponent monooxygenase, *Pseudomonas* sp. OX1 phenol hydroxylase. *Biochemistry* **45**, 15392–15404.

Sazinsky, M.H. and Lippard, S.J. 2006. Correlating structure with function in bacterial multicomponent monooxygenases and related diiron proteins. *Acc. Chem. Res.* **39**, 558–566.

Schafer, A., Tauch, A., Jager, W., Kalinowski, J., Thierbach, G., and Puhler, A. 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* **145**, 69–73.

Shingler, V., Powlowski, J., and Marklund, U. 1992. Nucleotide sequence and functional analysis of the complete phenol/3,4-dimethylphenol catabolic pathway of *Pseudomonas* sp. strain CF600. *J. Bacteriol.* **174**, 711–724.

Tabor, S. 2001. Expression using the T7 RNA polymerase/promoter system. *Curr. Protoc. Mol. Biol.* **11**, 16.12.11–16.12.11.