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NOTE

Characterization of Protocatechuate 4,5-Dioxygenase Induced from *p*-Hydroxybenzoate-Cultured *Pseudomonas* sp. K82

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***Pseudomonas* sp. K82 has been reported to be an aniline-assimilating soil bacterium. However, this strain can use not only aniline as a sole carbon and energy source, but can also utilize benzoate, *p*-hydroxybenzoate, and aniline analogues. The strain accomplishes this metabolic diversity by using different aerobic pathways. *Pseudomonas* sp. K82, when cultured in *p*-hydroxybenzoate, showed extradiol cleavage activity of protocatechuate. In accordance with those findings, our study attempted the purification of protocatechuate 4,5-dioxygenase (PCD 4,5). However the purified PCD 4,5 was found to be very unstable during purification. After Q-sepharose chromatography was performed, the crude enzyme activity was augmented by a factor of approximately 4.7. From the Q-sepharose fraction which exhibited PCD 4,5 activity, two subunits of PCD4,5 (α subunit and β subunit) were identified using the N-terminal amino acid sequences of 15 amino acid residues. These subunits were found to have more than 90% sequence homology with PmdA and PmdB of *Comamonas testosteroni*. The molecular weight of the native enzyme was estimated to be approximately 54 kDa, suggesting that PCD4,5 exists as a heterodimer ($\alpha_1\beta_1$). PCD 4,5 exhibits stringent substrate specificity for protocatechuate and its optimal activity occurs at pH 9 and 15°C. PCR amplification of these two subunits of PCD4,5 revealed that the α subunit and β subunit occurred in tandem. Our results suggest that *Pseudomonas* sp. K82 induced PCD 4,5 for the purpose of *p*-hydroxybenzoate degradation.**

Key words: *p*-hydroxybenzoate, protocatechuate 4,5-dioxygenase, *Pseudomonas* sp. K82

Protocatechuate 4,5-dioxygenase (PCD 4,5) is a major extradiol dioxygenase which facilitates the aerobic degradation of aromatic compounds in bacteria. PCD 4,5 catalyzes the extradiol cleavage of protocatechuate, producing 2-hydroxy-4-carboxy-muconic semialdehyde, which is ultimately converted into oxaloacetate and pyruvate by the *meta* pathways (Fig. 1). Various monocyclic (hydroxybenzoate, phthalate, vanillate, and *p*-toluate) and polycyclic (anthracene, phenanthrene, and naphthalene) aromatic compounds were reported to be channeled into PCD 4,5 for degradation (Providenti *et al.*, 2001). However, because of the instability of the enzyme *in vitro*, only a few PCD 4,5 has been purified and studied. This is unfortunate, as PCD4,5 is crucial in the microbial biodegradation of aromatic pollutants and lignin-derived compounds (Ono *et al.*, 1970; Arciero *et al.*, 1990; Chen *et al.*, 1994; Providenti *et al.*, 2001).

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Pseudomonas sp. K82 has previously been considered to be strictly an aniline-assimilating soil bacterium (Kanhg *et al.*, 1992). The *meta* cleavage pathway, which utilizes catechol 2,3-dixoygenase, has classically been listed as the prevalent pathway for aniline metabolism (CD 2,3). However, *Pseudomonas* sp. K82 exhibits another extradiol cleavage activity for *p*-hydroxybenzoate activity of PCD 4,5 (Kim *et al.*, 2004 personal communication). In order to obtain a biochemical understanding of *p*-hydroxybenzoate degradation in *Pseudomonas* sp. K82, we plan to purify and clone the genes which code for the degradation of *p*-hydroxybenzoate. The initial procedure in this study was the identification and characterization of PCD 4,5 from a hydroxybenzoate culture of *Pseudomonas* sp. K82.

Pseudomonas sp. K82 was pre-cultured at 28°C in potassium phosphate buffer (pH 6.25) containing 3.4 mM $MgSO_4$, 0.3 mM $FeSO_4$, 0.2 mM $CaCO_3$, 10 mM NH_4Cl and 10 mM sodium succinate, and then cultured in the same medium containing *p*-hydroxybenzoate (5 mM) for activity assay and characterization of enzyme. The bac-

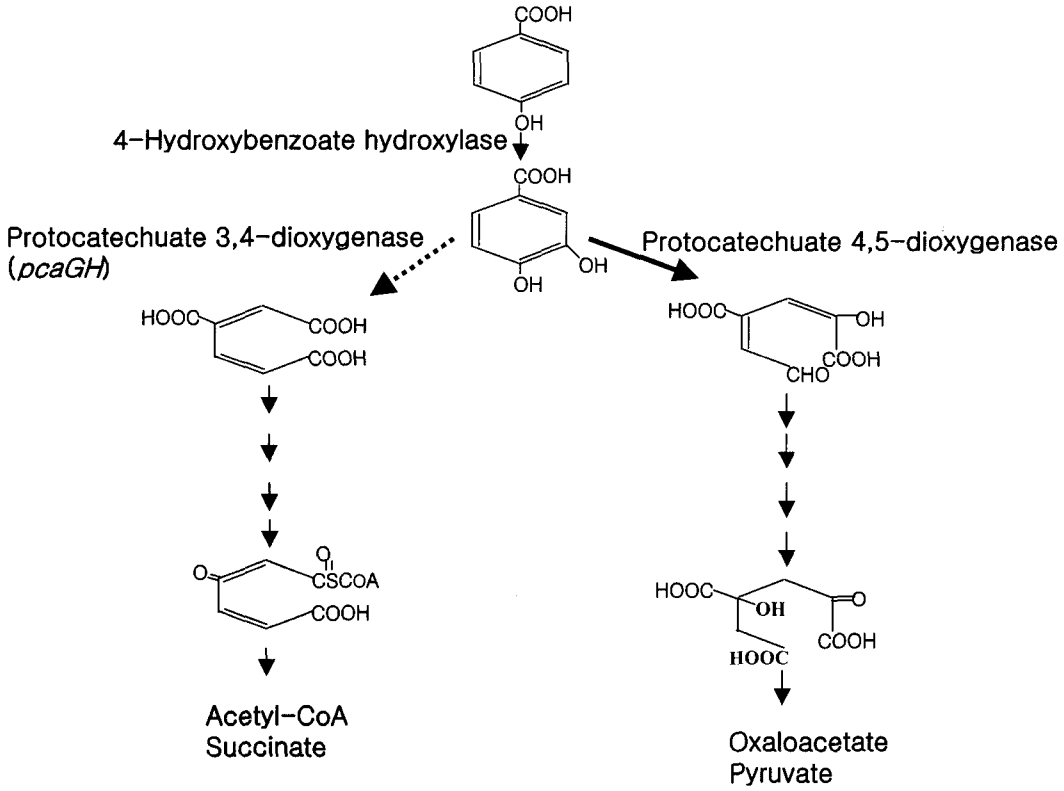

Fig. 1. Biodegradation pathway of 4-hydroxybenzoate and protocatechuate. *Pseudomonas* sp. K82 were showed extradiol cleavage activity for protocatechuate.

Table 1. Purification steps of protocatechuate 4,5-dioxygenase from *Psuedomonas* sp. K82

Purification steps	Vol(ml)	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Fold
Crude extract	130	957.8	29.02	0.030	1.00
Ammonium sulfate 30-50%	13	181.6	6.54	0.036	1.19
Q-sepharose	6	24.6	3.43	0.140	4.70

Harvested cells were suspended in 20 mM Tris-HCl buffer (pH 8.0) and disrupted using a French pressure cell (SLM AMINCO, Urbana, IL, USA) at 20,000 lb/in^2. The supernatant (crude cell extract), obtained by centrifugation at 15,000 g for 45 min, was subjected to 30 to 55 % ammonium sulfate precipitation and then dialyzed against Tris-HCl buffer (50 mM, pH 7.0). The resulting enzyme solution was applied to a Q-Sepharose column (HiLoad 16/10) and eluted with a 100 to 500 mM NaCl gradient at a flow rate of 2 ml/min for 40 min. Active fractions were pooled, dialyzed against 20 mM Tris-HCl buffer (pH 8.0). One unit of enzyme activity was defined as the amount which produced 1 μmol of 2-hydroxy-4-carboxy-muconic semialdehyde per min. The amount of 2-hydroxy-4-carboxy-muconic semialdehyde was calculated from the molar extinction coefficient at 34,000 $cm^{-1}M^{-1}$ (Ono *et al.*, 1970). The protein contents were determined by the method of Bradford (1976).

teria were harvested at the stationary phase and stored at -80°C until use. Crude extracts were prepared, and partial purification of PCD4,5 was performed according to the method described in Table 2. The supernatants (crude cell extracts) were used for activity assays of major extradiol and intradiol dioxygenases. Enzyme activities of catechol 1,2-dioxygenase (CD1,2), catechol 2,3-dioxygenase (CD 2,3), protocatechuate 3,4-dioxygenase (PCD3,4), and protocatechuate 4,5-dioxygenase (PCD4,5) were measured spectrophotometrically as reported methods (Ono *et al.*, 1970; Bull and Ballou, 1981; Aoki *et al.*, 1984; Nakanishi *et al.*, 1991). 0.03 unit/mg of PCD4,5 activity was observed in the crude extract, but other dioxygenase activities were not detected, excepting a small amount of PCD3,4 activity (0.01< unit/mg). However, activity of PCD4,5 dramatically decreased during the purification process. This rapid loss of activity was known to be due to the oxidation of an active-site ferrous iron group (Ono *et al.*, 1970). A 4.7-fold upshift in enzyme activity (0.14 unit/mg) was observed after Q-sepharose column chromatography (Table 1). Partially purified PCD4,5 showed strict substrate specificity for protocatechuate (data not shown). From SDS-PAGE of Q-sepharose fractions, two subunits of PCD4,5 were detected. Molecular weights of

Table 2. Comparative analysis of characteristics of protocatechuate 4,5-dioxygenases

Cell	*Pseudomonas* (*Comamonas*) testosterone	*Pseudomonas* sp.	*Rhizobium leguminosarum*	*Pseudomonas* sp. K82
Culture	*p*-Hydroxybenzoate	Protocatechuate	*p*-Hydroxybenzoate	*p*-Hydroxybenzoate
Substrate specificity	Protocatechuate	Protocatechuate	Protocatechuate	Protocatechuate
Effect of Inhibitors	H_2O_2, Ferricyanide	KCN, H_2O_2, Sodium mersalyl,	-	H_2O_2
Optimal pH	8.0-8.5	7.0	9.5-9.8	8.8-9.0
Subunit Mw (Da)	α-17,700 β-33,800	-	α-62,000	α-17,000, β-34,000
Native Mw GPC (Da)	142,000 ($\alpha_2\beta_2$)	150,000	120,000 (α_2)	54,000 (αβ)
Km	46 μM	54 μM	20 μM	-
Effect of Metals	Fe^{+2} ↑ Mn^{+2} ↓, Co^{+2} ↓, Ni^{+2} ↓	Fe^{+2} ↑	-	Fe^{+2} ↑, Ni^{+2} ↑, Mg^{+2} ↑, Mn^{+2} ↓, Hg^{+2} ↓, Ag^{+2} ↓
Reactivation Agents	Fe^{+2}, Ascorbic acid	Fe^{+2}, Ascorbic acid	-	Fe^{+2}
References	Dagley *et al.*, 1963 Arciero *et al.*, 1990 Providenti *et al.*, 2001	Ono *et al.*, 1970	Chen *et al.*, 1994	This study
GenBank accession No	M34835	-	-	-

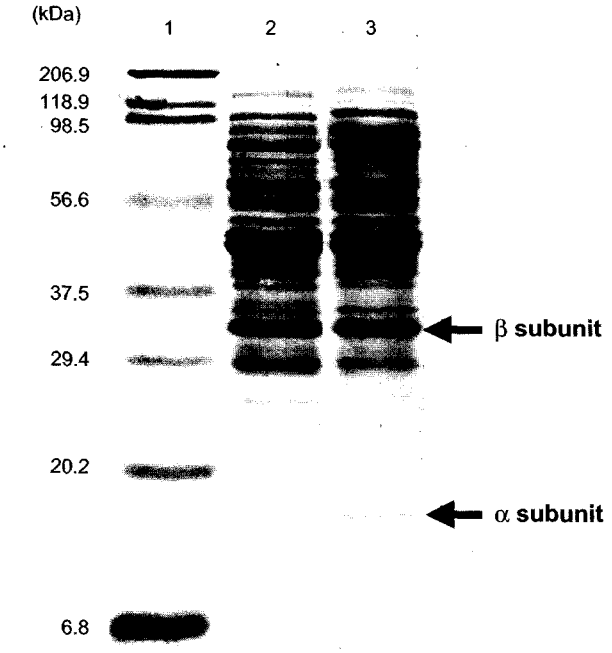

Fig. 2. Protocatechuate 4,5-dioxygenase of Q-sepharose fractions from *Pseudomonas* sp. K82 on a sodium dodecyl sulfate (SDS) polyacrylamide gel. Two subunits of PCD4,5 on the SDS-gel were transferred onto a PVDF membrane using a semi-dry blotting apparatus (BIO-RAD Trans-Blot SD) at 2 mA/cm^2 for 50 min. The PVDF membrane was stained with a Coomassie Brilliant blue R250 and washed with 60% methanol. Coomassie stained two subunit of PCD4,5 were excised from the PVDF membrane and installed into the blot cartridge of a model 491A protein sequencer (Perkin-Elmer, USA) for sequencing analysis. The obtained N-terminal sequence was used for protein identification by BLAST search of NCBI. Lanes: 1, standard markers; 2, Q-sepharose fraction 1; 3, Q-sepharose fraction 2. Subunit α and subunit β are indicated by arrows.

the two subunits (α- and β- subunit) were estimated to be 17 kDa for the α-subunit, and 34 kDa for the β- subunit (Fig. 2). N-terminal amino acid sequences of α- and β- subunit were analyzed using an Edman Sequanator ABI 491A (ALDKPYLDVPGTIIF and ARITASVYTSHV-PAI, respectively). These PCD4,5 subunits from *Pseudomonas* sp. K82 evidence a high degree of sequence homology with those isolated from *Comamonas testosteroni* (Providenti *et al.*, 2001), *Pseudomonas straminea* (accession no. BAD0457), and *Rhodopseudomonas palustris*. (accession no. NP_950035 and NP_950036). Additionally, MS/MS analysis showed that the internal amino acid sequences of PCD4,5 were very similar to those of PmdAB from *P. testosteroni* (Kim *et al.*, 2004 personal communication). Sequencing data from the *P. testosteroni* study can also be used for the cloning of PCD4,5 from *Pseudomonas* sp. K82. Sequence analysis revealed that the N-terminal methionines of the two subunits had been removed. This suggests that methionyl aminopeptidase was working in *Pseudomonas sp.* K82 to remove alanine. The molecular weight of native enzyme was estimated to be approximately 54 kDa by GPC column (Sephadex 200), suggesting that the enzyme exists as a heterodimeric unit (αβ) in the cell (data not shown). Characteristics of PCD4,5 from *Pseudomonas* sp. K82 are summarized and compared with other enzymes in Table 2. Effects of PCD4,5 for metals or inhibitors (Fe^{+2}, Mn^{+2} and H_2O_2) are similar to those exhibited by the PmdAB of *P. testosteroni.* However, the optimal pH range for PCD4,5 (pH 8.8 - 9.0) is between the optimal pH ranges of *P. testosteroni* and *R. leguminosarum.* Also, PCD4,5 optimal activity occurred at an unexpectedly low temperature (15°C) (Fig. 3). This is a curious result, as the growth rate of *Pseudomonas* sp. K82 at 15°C is 4.0 times slower than the growth rate at 28°C. PCR gene amplification was the preliminary step in the cloning of PCD4,5. Oligomers were

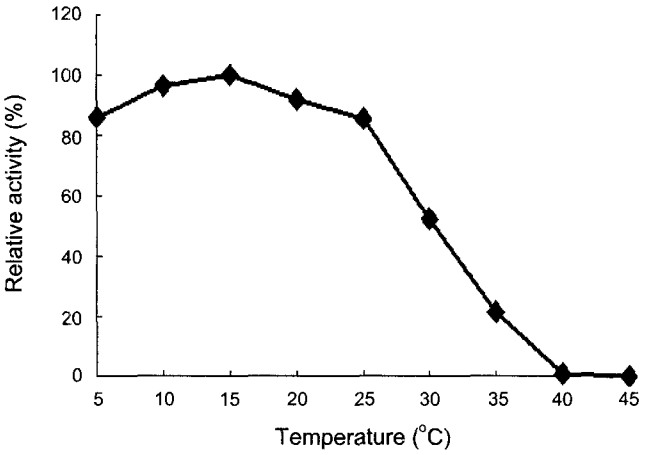

Fig. 3. Effect of temperature on the activity of PCD4,5 from *Pseudomonas* sp. K82.

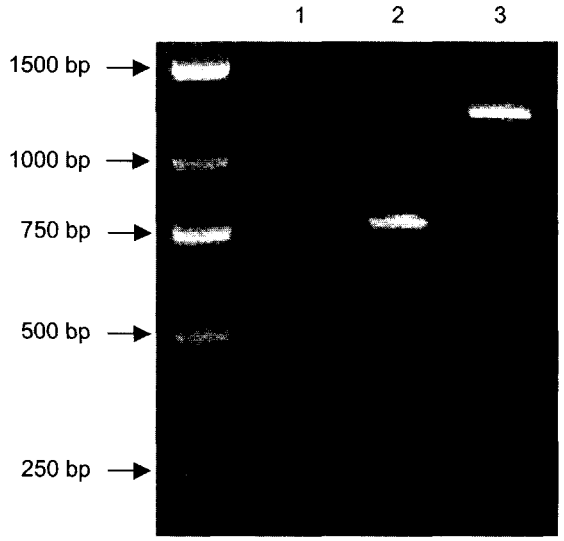

Fig. 4. PCR amplification of two subunits of PCD4,5 from *Pseudomonas* sp. K82. Lanes: 1, molecular size marker; 2, PCR product (240 bp) of α. subunit; 3, PCR product (750 bp) of β subunit; 4, PCR product (1,200 bp) of αβ subunit.

designed using N-terminal and internal amino acid sequences, as follows: α subunit-F1, AAA CCG TAT CTG GAC GTG CCC GGC A (KPYLDVPGT); α subunit-R1, CAG GAA GTA GAT ATT GCC GCC GGT (TGGNIYFL); β subunit-F2, ATG GCT CGC ATC ACC GCA TCC GTT (MARITASV); β subunit-R2, GGC ACC ACG CGC AAT CAG CCA CAT (MWLIARGA). 240 bp products were amplified using the oligomers of the α subunits F1 & R1, and 750 bp PCR products were amplified using the oligomers of the β subunits F1 & R2. (Fig. 4). A sequence of approximately 1200 bp was amplified by PCR using F1 & R2. These results suggest that the two subunits of PCD4,5 were located in tandem. In general, two subunits of PCD4,5 were co-translated in reported bacteria (Noda *et al.*, 1990; Providenti *et al.*, 2001). These PCR products can be used in *Pseudomonas* sp. K82 as probes for the cloning of the gene cluster which controls *p*-hydroxybenzoate biodegradation. In summary, although *Pseudomonas* sp. K82 has the ability to use several dioxygenase systems, this strain utilizes PCD4,5 as its main biodegradative dioxygenase only when cultured in *p*-hydroxybenzoate. The interrelationship of dioxygenases in *Pseudomonas* sp. K82 will be the subject of future studies.

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