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Characteristics of Catechol 2,3-Dioxygenase Produced by 4-Chlorobenzoate-degrading *Pseudomonas* sp. S-47

Ki-Pil Kim, Dong-In Seo, Kyung-Hee Min[1], Jong-Ok Ka[1], Yong-Keun Park[1], and Chi-Kyung Kim*

Department of Microbiology, Chungbuk National University, Cheongju 361-763, and [1]Research Center for Molecular Microbiology, Seoul National University, Seoul 151-742, Korea

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***Pseudomonas* sp. S-47 is capable of transforming 4-chlorobenzoate to 4-chlorocatechol which is subsequently oxidized by *meta*-cleavage dioxygenase to produce 5-chloro-2-hydroxymuconic semialdehyde. Catechol 2,3-dioxygenase (C23O) produced by *Pseudomonas* sp. S-47 was purified and characterized in this study. The C23O enzyme was maximally produced in the late logarithmic growth phase, and the temperature and pH for maximum enzyme activity were 30~35°C and 7.0, respectively. The enzyme was purified and concentrated 5 fold from the crude cell extracts through Q Sepharose chromatography and Sephadex G-100 gel filtration after acetone precipitation. The enzyme was identified as consisting of 35 kDa subunits when analyzed by SDS-PAGE. The C23O produced by *Pseudomonas* sp. S-47 was similar to XylE of *Pseudomonas putida* with respect to substrate specificity for several catecholic compounds.**

Key words: Catechol 2,3-dioxygenase (C23O), 4-chlorobenzoate degradation, *Pseudomonas* sp. S-47

Aromatic hydrocarbons and chlorinated aromatics are recognized to be major groups of pollutant chemicals because of their recalcitrance against degradation and their toxicity to human and wild lives (7, 8). Among them, biphenyl, polychlorinated biphenyls (PCBs), chlorophenol, chlorobenzene, and chlorophenoxyacetate have been extensively studied with regards to their degradation by microorganisms. However, biodegradation of these chemicals is reported to be limited due to their stringent specificity for substrates, steps of metabolic pathway, and environmental conditions (1, 7).

The biodegradation of aromatic hydrocarbons under aerobic conditions is mostly conducted by oxidative reactions to produce catechol or chlorocatechol as common intermediates which are ultimately metabolized for carbon and energy sources via the tricarboxylic acid cycle (13, 18, 22). The oxidative degradation of catechol and catecholic compounds are catabolized by catechol dioxygenases via either *meta*-or *ortho*-cleavage of the benzene structure (6, 7, 22). The *meta*-cleavage (extradiol) dioxygenases are classified as a superfamily of catechol 2,3-dioxygenase (C23O, metapyrocatechase, catechol-oxygen 2,3-oxidoreductase, EC 1.13.1.2) which is encoded by *xylE* of TOL plasmids and pWWO, *nahH* of NAH7 plasmid in *P. putida* (4, 5), and *todE* (23, 24) and *bphC* in *Pseudomonas* spp. (10, 13 21). Catechol 2,3-dioxygenase which catalyzes the *meta*-cleavage of catechol and catecholic compounds was reported to be a homo-octamer consisting of 35 kDa subunits that contain ferrous ion as a functional cofactor (15, 17).

Pseudomonas sp. S-47 is a bacterial strain isolated from the contaminated wastes in our laboratory. The strain is capable of transforming 4-chlorobenzoate aerobically to 4-chlorocatechol (4CC) which is subsequently oxidized by *meta*-cleavage dioxygenase to produce the yellow-colored *meta*-cleavage product, 5-chloro-2-hydroxymuconic semialdehyde (5C-2HMS), as shown in Fig. 1 (19). In this study, the catechol 2,3-dioxygenase (C23O) produced by *Pseudomonas* sp. S-47 which catalizes the *meta*-cleavage reaction of 4CC to 5C-2HMS was purified and characterized for degradative activity on catecholic compounds.

Materials and Methods

Bacterial strain and growth

Pseudomonas sp. S-47 is a bacterial strain isolated from the contaminated wastes in this laboratory as reported by Seo *et al.* (19). The or-

* To whom correspondence should be addressed.

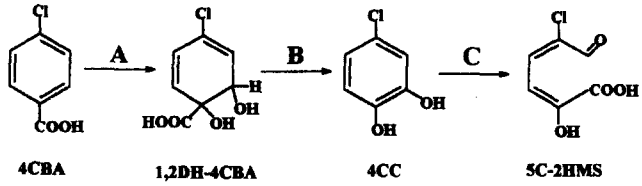

Fig. 1. Pathway for degradation of 4-chlorobenzoate via 4-chlorocatechol *meta*-cleavage by *Pseudomonas* sp. S-47. A, benzoate dioxygenase; B, benzoate diol dehydrogenase; C, catechol 2,3-dioxygenase. Abbreviation: 4CBA, 4-chlorobenzoate; 1,2DH-4CBA, 1,2-dihydro-4-chlorobenzoate; 4CC, 4-chlorocatechol; 5C-2HMS, 5-chloro-2-hydroxymuconic semialdehyde.

ganisms were cultivated in Luria-Bertani (LB) broth medium with a shaking incubator at 30°C.

Purification of C23O

The cells were collected from the LB broth culture after 20 hours of growth and suspended in a 10 mM potassium phosphate buffer (pH 7.0). To prepare crude cell extract, the cells were cracked with a sonicator (Fisher M-300, Pittsburgh, U.S.A.). The extract was mixed with cold acetone (−20°C) to precipitate proteins and centrifuged at 12,000×*g* to collect precipitates according to the procedures described by Bollag *et al.* (3). The precipitates, which were resuspended in 10 mM Tris-HCl buffer (pH 8.0) for 24 hours, were adsorbed in the Q sepharose ion exchange column (Lot No. 228638, Pharmacia, Sweden), washed with the same buffer, and then fractionated in 5 ml guantities with the GP-250 FPLC (fast protein liquid chromatography) system by flowing through a 0 to 1.5 M NaCl gradient (12). The total protein of each fraction was measured at 280 nm with a UV-visible spectrophotometer and the activity of C23O on 4CC was assayed by the procedures described by Kim *et al.* (14). The fractions showing C23O activity were collected and the C23O enzyme was concentrated with Amicon Centricon-50 microconcentrator (Amicon, Beverly, U.S.A.) and then filtered through a Sephadex G-100 gel (bead diameter, 20 μm; Lot No. HB-24556, Pharmacia, Uppsala, Sweden) which was equilibrated with 10 mM Tris-HCl buffer (pH 8.0) by the methods described by Bollag *et al.* (3) and Sung *et al.* (20).

Assay for specific activity of C23O

The activity of C23O on 4CC and other aromatic compounds was examined by resting cell assay at 30°C and pH 7.0 according to the methods described by Kim *et al.* (14). Ten microliters of the enzyme sample and 50 μl of substrates were reacted by mixing together in 940 μl of 10 mM potassium phosphate buffer (pH 7.0). The *meta*-cleavage product (MCP) produced from each substrate by C23O was then examined. The MCP produced from 4CC (ε=36.9/cm/M) was measured at 379 nm. One unit of the activity was defined as 1 μmol MCP produced per minute.

The effects of temperature and pH on the activity of C23O were assayed in the same way as described above. The pH of the reaction buffer was adjusted with 10 mM potassium phosphate buffer for pH range 6.0 to 8.0 and with 10 mM glycine-NaOH buffer for pH 8.0 to 10.0.

SDS-PAGE

SDS-PAGE of the C23O enzyme was carried out using 10 and 4% acrylamide for separation and stacking gels, respectively. The gels were electrophoresed with running buffer (0.025 M Tris, 0.192 M glycine, 0.1% SDS, pH 8.3) at 50 mA for 4 hours, stained with a staining solution (0.025% Coomassie Blue, 40% methanol, 7% acetic acid) for 3 hours, and then destained with solution I and solution II for 1 and 10 hours, respectively, by the method of Bollag *et al.* (3).

Test for substrate specificity of C23O

The substrate specificity of C23O was examined for catechol, 3-chlorocatechol (3CC), 4-chlorocatechol (4CC), 3-methylcatechol (3MC), 4-methylcatechol (4MC), and 2,3-dihydroxybiphenyl (2,3-DHBP) in the same methods as described above (14). The *meta*-cleavage products (MCPs) produced from the substrates by the enzyme were measured by a UV-visible spectrophotometer and the specific activity of C23O calculated using the molar extinction coefficients of the substrates. The MCPs produced from catechol (ε=33,000/cm/M), 4MC (ε=28,100/cm/M), 3MC (ε=13,400/cm/M), 3CC (ε=33,000/cm/M), and 2,3-DHBP (ε=22,000/cm/M) were measured at 375 nm, 379 nm, 388 nm, 375 nm and 434 nm, respectively (2, 13).

Results and Discussion

Production and purification of C23O

The production of C23O by *Pseudomonas* sp. S-47 during its growth is shown in Fig. 2. When *Pseudomonas* sp. S-47 inoculated at a concentration of 10^7 CFU/ml in LB broth was cultivated at 30°C, the exponential phase was extended up to 28 hours of incubation. The activity of C23O also gradually increased during exponential phase, and decreased as stationary phase began. Therefore, cells grown for 20 to 25 hours in LB broth was used for purification of the C23O enzyme.

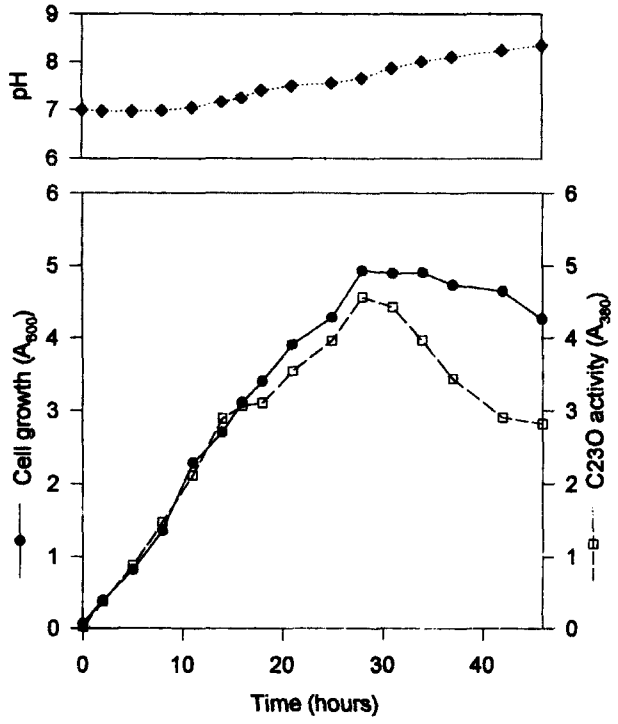

Fig. 2. Growth and catechol 2,3-dioxygenase activity of *Pseudomonas* sp. S-47. *Pseudomonas* sp. S-47 was cultured in LB broth at 30°C.

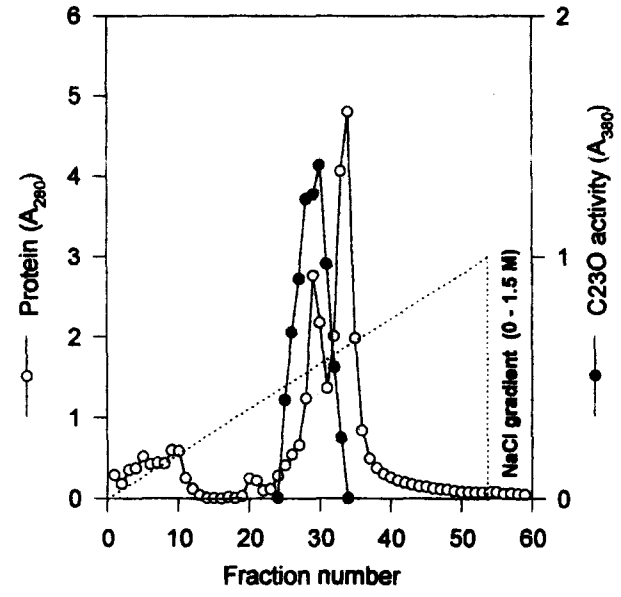

Fig. 3. Q Sepharose chromatogram and C23O activity of acetone fraction proteins of *Pseudomonas* sp. S-47.

Total proteins and C23O activity of the fractions eluted from the Q sepharose column are shown in Fig. 3. Among the fractions, the samples (No. 24 to 34) obtained within the range of 0.7 to 0.9 M NaCl exhibited C23O activity. These samples were pooled, concentrated, and then filtered through dextran gel. The amount and C23O activity of total proteins as well as the specific activity of the C23O in each purification step are shown in Table 1. The recovery yield of C23O after gel filtration was 0.7% of total protein in crude extract and a five-fold purification of C23O was obtained.

SDS-PAGE confirmed that the C23O produced by *Pseudomonas* sp. S-47 consisted of 35 kDa subunits as shown in lanes B to D in Fig. 4. The XylE dioxygenase subunit produced by *Pseudomonas putida* mt-2 was reported to be 32 kDa in molecular mass (15), but C23O (TomB) expressed by TOM plasmid cloned from *Pseudomonas cepacia* G4 was 35 kDa (17). This means that the enzyme has been differentiated in molecular mass among the strains isolated from geologically different and polluted environments.

Dioxygenase activity and substrate specificity

The dioxygenase activity of C23O on 4CC was examined by resting cell assay for production of the *meta*-cleavage product (5M-2HMS) detecting at 379 nm. The optimum temperature and pH for C23O activity were 30~35°C and 7.0, respectively, as shown in Fig. 5. Cerdan *et al.* (4) reported that specific activity of C23O on catecholic compounds can be affected by various environmental stresses. This means that the potential C23O activity of *Pseudomonas* sp. S-47 might also be developed by contaminant aromatic pollutants considering the location of its isolation as reported by Seo *et al.* (19).

Table 1. Catechol 2,3-dioxygenase activity of *Pseudomonas* sp. S-47 in each step of purification

Purification step	Total activity (unit)	Total protein (mg)	Recovery yield (%)[a]	Specific activity (unit/mg)	Purification fold[b]
Crude Extract	2460.0	109.0	100.0	22.6	1.0
Acetone precipitaion	1260.0	45.0	41.3	28.0	1.2
Q sepharose chromatography	183.0	3.6	3.3	50.8	2.2
Sephadex G-100 filtration	8.6	0.8	0.7	113.8	5.0

[a] Recovery yield is the percentage of total protein to that of crude extract.
[b] Purification fold is the fold of specific acitivity of C23O in crude extract.

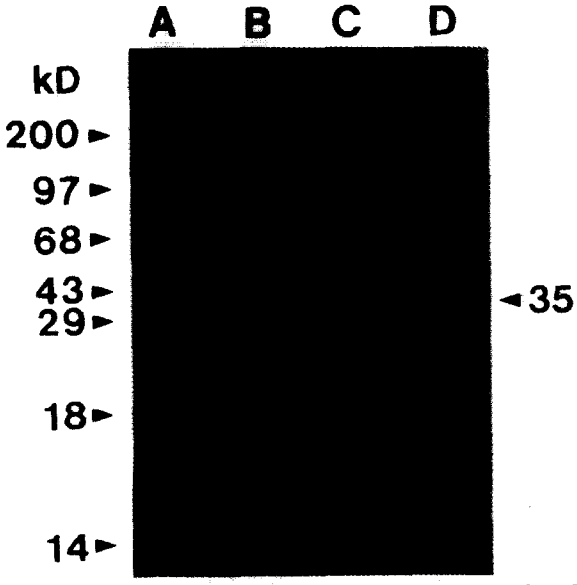

Fig. 4. SDS-PAGE of catechol 2,3-dioxygenase produced by *Pseudomonas* sp. S-47. Lanes: A, size marker; B, C, and D, C23Os produced by *Pseudomonas* sp. S-47.

The substrate specificities of C23O for several catecholic compounds and 2,3-dihydroxybiphenyl are shown in Table 2. The C23O enzyme produced by *Pseudomonas* sp. S-47 revealed the most potential specific activity for catechol. The activities of C23O on 4CC, 4MC, and 3MC were 71.4, 62.8, and 33.7%,

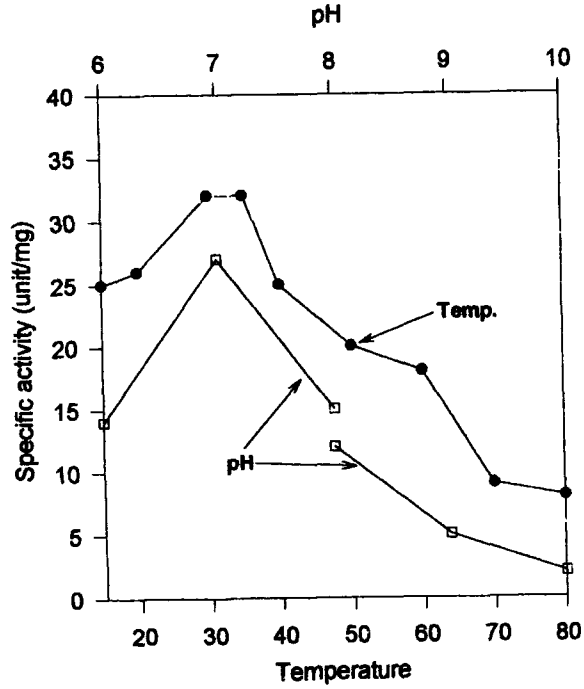

Fig. 5. Specific activity of catechol 2,3-dioxygenase at different temperature and pH.

Tabel 2. Substrate specificity of catechol 2,3-dioxygenase produced by *Pseudomonas* sp. S-47[a]

Substrate	Specific activity (unit/mg)	Relative activity (%)
Catechol	31.5	100
4-Chlorocatechol	22.5	71.4
4-Methylcatechol	19.8	62.8
3-Chlorocatechol	0.5	1.6
3-Methylcatchol	10.6	33.7
2,3-Dihydroxybiphenyl	1.9	6.0

[a] The substrate specificity of catechol 2,3-dioxygenase was determined with each substrate under the standard enzyme assay condition as described in the materials and

respectively, compared with the activity for catechol. However, the activities of C230 on 3CC and 2,3-DHBP were only 1.6 and 6.0%, respectively. The activity difference between substrates could be explained by the radicals and position of substitution as reported by Cerdan *et al.* (5). Such low activity of C23O on 3CC is considered to be due to the inactivation effect of the metabolite reported by Bartels *et al.* (2). Bartels *et al.* (2) found that acyl halide produced from 3CC inactivates the C23O en-

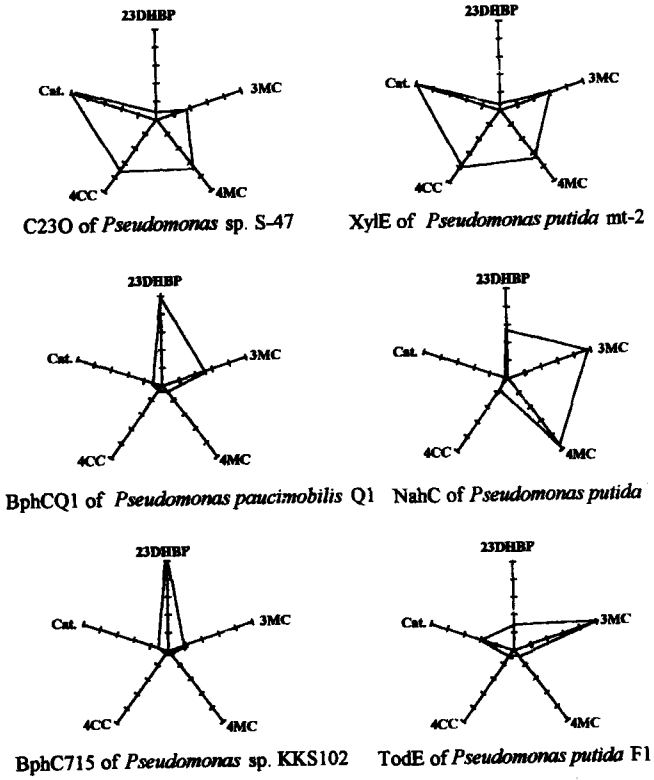

Fig. 6. Comparison of substrate specificity of C23O produced by *Pseudomonas* sp. S-47 with those reported in other strains. Abbreviation: 23DHBP, 2,3-dihydroxybiphenyl; Cat., catechol; 4CC, 4-chlorocatechol; 4MC, 4-methylcatechol; 3MC, 3-methylcatechol.

zyme in *Pseudomonas putida* mt-2. A comparison of the substrate specificity of C23O produced by *Pseudomonas* sp. S-47 with those of the same *meta*-cleavage (extradiol) dioxygenases reported in other *Pseudomonas* strains was made in Fig. 6 as originally figured by Hirose *et al.* (11). The *meta*-cleavage dioxygenase (C23O) produced by *Pseudomonas* sp. S-47 was very similar to XylE (16) of *Pseudomonas putida* mt-2 in terms of substrate specificity for catacholic compounds, but quite different from BphC (10, 21), NahC (9), and TodE (24) which exhibits potential *meta*-cleavage activity on 2,3-DHBP, 3MC and 4MC, and 3MC, respectively.

Acknowledgments

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