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Purification and Characterization of an Extradiol Dioxygenase Which Preferentially Acts on 4-Methylcatechol

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Abstract A catechol 2,3-dioxygenase (C23O) was purified to apparent homogeneity from *Pseudomonas putida* SU10 through several purification steps consisting of ammonium sulfate precipitation and chromatographies on DEAE 5PW, Superdex S-200, and Resource-Q. Gel filtration indicated a molecular mass under nondenaturing conditions of about 130 kDa. The enzyme has a subunit of 34 kDa as was determined by SDS-PAGE. These results suggest that the native enzyme is composed of four identical subunits. The N-terminal amino acid sequence (30 residues) of the enzyme has been determined and exhibits high identity with other extradiol dioxygenases. The reactivity of this enzyme towards catechol and methyl-substituted catechols is somewhat different from that seen for other catechol 2,3-dioxygenases, with 4-methylcatechol cleaved at a higher rate than catechol or 3-methylcatechol. K_m values of the enzyme for these substrates are between 3.5 and 5.7 M.

Key words: Catechol 2,3-dioxygenase, substrate specificity, N-terminal sequence, kinetic studies, *Pseudomonas putida* SU10

Dioxygenases are involved in a variety of reactions, including the cleavage of aromatic rings. Non-haem iron dioxygenases are the bacterial enzymes which cleave the double bonds of aromatic compounds adjacent to hydroxyl groups [21, 24]. These include the catechol dioxygenases, which divide into Fe(III)-containing enzymes which cleave in an intradiol fashion and Fe(II)-containing enzymes which cleave in an extradiol fashion [21, 24]. It was previously believed that the site of cleavage of an aromatic ring is strictly specific for each enzyme, which cleaves the catechol ring in the intradiol manner or in the extradiol manner [19, 20].

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Catechol 2,3-dioxygenase (C23O) isolated from *Pseudomonas putida* mt-2 (*arvilla*) and grown on benzoate as a sole carbon source and an inducer, contains Fe(III) as a cofactor [20] and cleaves catechol in an extradiol fashion to a semialdehyde [19]. This enzyme had a molecular weight of 140 kDa, but showed a single band of a subunit with a molecular mass of 35 kDa on SDS-PAGE gel, indicating that the enzyme consists of four identical subunits [17, 24]. It has also been reported that catechol 2,3-dioxygenases from *P. putida* KT2440 and *P. putida* ATCC 23973 also consist of four identical subunits, each containing one ferrous ion [4, 24].

McClure and Venables [13, 14] have described a *Pseudomonas putida* UCC2 able to utilize *m*- or *p*-toluidines as the sole carbon and nitrogen source. This UCC2 strain expresses a novel extradiol dioxygenase which is plasmid-encoded [14]. Kinetic parameters of catechol 2,3-dioxygenase showed a broad substrate specificity for catechol, 4-methylcatechol, and 4-chlorocatechol. Walls and Chapman [24] reported the purification and partial characterization of 3-methylcatechol 2,3-dioxygenase which preferentially acts on 3-methylcatechol rather than catechol. The reactivity of this enzyme towards catechol and methyl-substituted catechols showed a higher cleavage rate at 3-methylcatechol than catechol or 4-methylcatechol which is somewhat different from other catechol 2,3-dioxygenases. The enzyme catalyzes the ring cleavage of catechol and 3-methylcatechol, but shows weak activity towards 4-methylcatechol [4]. Partially purified catechol 2,3-dioxygenase from toluene-grown cells of *P. putida* catalyzed catechol, 3-methylcatechol, 4-methylcatechol, and 4-fluorocatechol [15]. The enzyme exhibited greater affinity and activity for 3-methylcatechol than for catechol [10].

The reported homologies between the amino acid sequences among C23O which catalyze the extradiol cleavage of an aromatic ring were highly similar to those of other *meta*-cleavage enzymes [3], despite of their

broad specificty for catechol, 3-methylcatechol, and 4-methylcatechol.

In the present paper, we report the purification and special characteristics of catechol 2,3-dioxygenase from *P. putida* SU10, which preferentially acts on 4-methylcatechol more than on catechol or 3-methylcatechol. We examined a comparison of N-terminal amino acid sequences of the enzymes to the well characterized extradiol dioxygenases from other bacterial sources.

MATERIALS AND METHODS

Bacterial Strain and Growth Conditions

The bacterial strain, *Pseudomonas putida* SU10, used in this study was isolated from sewage with *p*-toluic acid as a major carbon source [22]. The bacterial cells were grown in a medium containing 0.44% K_2HPO_4, 0.34% KH_2PO_4, 0.2% $(NH_4)_2SO_4$, and 5 mM *p*-toluic acid (pH 7.5) as a sole carbon source. Cultivations were carried out in one liter Erlenmeyer flasks containing 200 ml of the sterilized liquid media at 30°C for 24 h with a rotary shaker (180 rpm). The cells were harvested with a centrifuge at 10,000×g for 90 min.

Assay for Catechol 2,3-Dioxygenase Activity

The catechol 2,3-dioxygenase (C23O) activity was measured spectrophotometrically in 50 mM phosphate buffer (pH 7.5) containing 0.5 mM substrate at 30±0.1°C. One unit of the enzyme activity was defined as the amount of enzyme that converts 1 μmol of substrate to *meta*-cleavage compound per minute. The λ_{max} values of the cleavage compounds of each substrate were as follows: 382 nm for 2-hydroxy-5-methyl-6-oxohexa-2,4-dienoic acid (4-methylcatechol), 375 nm for hexa-2,4-dienedioic acid (catechol), 380 nm for 2-hydroxy-6-oxohepta-2,4-dienoic acid (3-methylcatechol), 432 nm for 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (dihydroxybiphenyl), 380 nm for 5-carboxy-methyl-2-hydroxy-*cis,cis*-muconic semialdehyde (homo-protocatechuic acid), and 410 nm for α-hydroxy-γ-carboxymuconic semialdehyde (protocatechuic acid). The molar concentration of each compound was calculated from the observed absorbance at λ_{max} and the corresponding molar extinction coefficient [8, 9, 11, 24]. The specific activity of the enzyme was defined as unit(s) per mg of protein.

Determination of Protein Concentration

Protein concentration was determined by the method of Lowry *et al.* [12] using bovine serum albumin as the reference protein.

Purification of 4-Methylcatechol 2,3-Dioxygenase

Frozen cells (50 g) were thawed and suspended in 100 ml of 50 mM phosphate buffer (pH 7.5) containing 10% acetone. Cells were broken by exposure for 60 min to the output of a ultrasonic processor (Danbury, U.S.A.). Crude extracts, containing 15 mg of protein per ml, were obtained by centrifugation at 12,000×g for 30 min, treated with 70% ammonium sulfate. The mixture was stirred at 4°C for 30 min and the precipitate was removed by centrifugation. After standing overnight, the precipitate was collected by centrifugation, dissolved in 10 ml of supplemented phosphate (pH 7.5), and dialyzed for 24 h at 4°C. The dialyzed enzyme was then chromatographed on a DEAE-5PW column (2.15×15 cm) equilibrated with supplemented phosphate buffer (pH 7.5). The column was washed with the buffer and eluted with a linear gradient of 0 to 0.5 M NaCl in a total of 500 ml of the buffer. The flow rate was 5 ml/min and 5 ml fractions were collected. Fractions having enzyme activities were mixed together, and concentrated with Amicon (Millipore, Bedford, U.S.A.). All chromatography procedures were carried out at 4°C.

This enzyme solution was then applied to a column of Superdex S-200 (1.6×60 cm) preequilibrated with the same buffer. The flow rate was 1ml/min and 1 ml fractions were collected. Fractions with high activities were mixed, followed by concentration with Amicon. Finally, the enzyme solution was applied to a column of Resource-Q (Sigma, St. Louis, U.S.A.) with a 6 ml volume in an FPLC system (Pharmacia, Uppsala, Sweden) equilibrated with the same buffer and eluted with a lear gradient of 0 to 0.4 M NaCl at the flow rate of 1 ml/min (1.0 ml per fraction).

Determination of Molecular Mass

The molecular mass of the native enzyme was estimated using gel filtration on a Superdex S-200 column calibrated with the following molecular markers; ferritin (450 kDa), beef liver catalase (240 kDa), lamb muscle aldolase (158 kDa), bovine serum albumin (68 kDa), egg albumin (45 kDa), chymotrypsin A (25 kDa), and cytochrome C (12.5 kDa). SDS-polyacrylamide gel electrophoresis was carried out to estimate the subunit molecular weight of C23O purified from *P. putida* SU10. A calibration mixture of myosin (200 kDa), β-galactosidase (116.3 kDa), phosphorylase (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and trypsin inhibitor (21.5 kDa) was applied on Tricine/SDS/polyacrylamide gel electrophoresis. These marker proteins were purchased from Boehinger Mannheim (Mannheim, Germany).

N-Terminal Amino Acid Sequencing

The N-terminal sequence was determined by automated Edman degradation with a Miligen/Biosearch 6600 Prosequencer system (Millipore, Milford, U.S.A.) at the Korea Basic Science Institute, Taejon, Korea.

Kinetic Studies

All measurements for kinetic studies including pH and temperature effect were carried out under steady-state

conditions with the substrate. Kinetic experiments were carried out in 50 mM phosphate buffer at pH 7.5 and at 30±0.1°C with the substrate, catechol, 3-methylcatechol, 4-methylcatechol, and dihydroxybiphenyl. Enzyme activities were measured by determining the reaction products (v_0: initial velocity) using the Perkin-Elmer λ9 spectrophotometer (Norwark, U.S.A.). Experimental data were fitted to a non-linear least-squares program and also represented as Lineweaver-Burk plots from Michaelis-Menten curves to yield K_m and V_{max} values.

RESULTS AND DISCUSSION

Purification of C23O

The crude extract of catechol 2,3-dioxygenase (C23O) was sequentially purified by ammonium sulfate precipitation, DEAE 5PW chromatography, Superdex S-200 gel filtration, and Resource-Q chromatography. Elution profiles of the last two steps during chromatography are shown in Fig. 1. On the elution profile of Resource-Q ion-exchange chromatography, the unique activity peak coincided with a protein peak. Purity of each elution during the purification process was examined by electrophoresis on a 12% SDS-PAGE (Fig. 2C) indicating that C23O from *P. putida* SU10 was in a pure state. The active fraction from Resource-Q ion-exchange chromatography showed a single protein band (Fig. 2C, lane 3) on SDS-PAGE. The purification procedure of the enzyme is summarized in Table 1. Purification fold and yield were 27.2 and 49%, respectively.

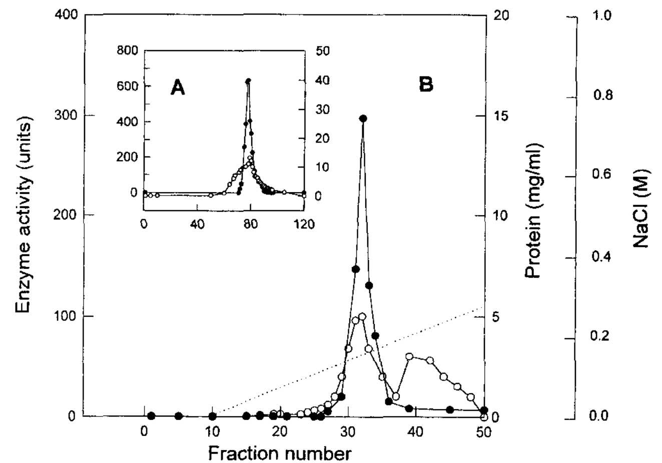

Fig. 1. Purification of catechol 2,3-dioxygenase on Superdex S-200 gel filtration (A) and on Resource-Q ion-exchange column chromatography (B).
Symbols: (○), protein concentration; (●), enzyme activity, and dashed line (----) in B indicates the gradient concentration of sodium chloride.

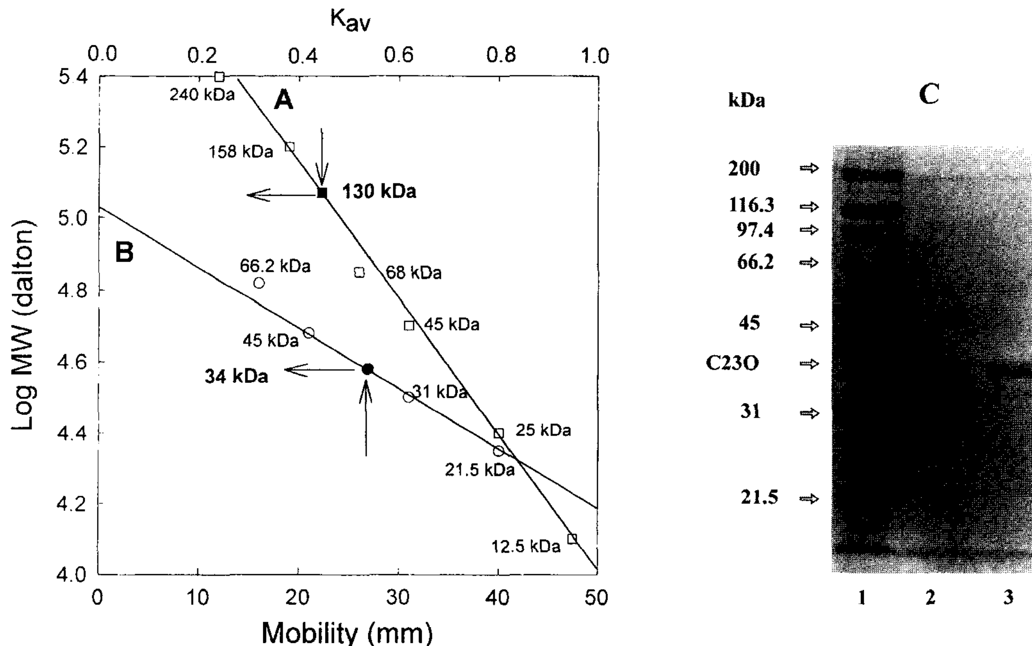

Fig. 2. (A) Estimation of molecular weight of native C23O by gel filtartion on Superdex S-200 column.
The molecular weights of marker proteins are designated beside the open symbols. The size marker proteins were: beef liver catalase (240 kDa), lamp muscle aldolase (158 kDa), bovine serum albumin (68 kDa), hen-egg albumin (45 kDa), chymotrypsin A (25 kDa), cytochrome C (12.5 kDa). $K_{av}=(V_e-V_0)/(V_t-V_0)$, where V_e, V_0, and V_t are the elution volume, void volume, and the total column volume, respectively. (B) and (C). Determination of the molecular weight of the subunit of purified C23O on SDS-PAGE. Lane 1 in C represents size markers; myosin (200 kDa), β-galactosidase (116.3 kDa), phosphorylase (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and trypsin inhibitor (21.5 kDa). Lanes 2 and 3 in C are the protein after Superdex S-200 gel filtration and the purified enzyme after Resource-Q ion-exchange column chromatography, respectively.

Table 1. Purification scheme of catechol 2,3-dioxygenase.

Step	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Yield (%)	Purification fold
Crude extract	2,400	-	-	-	-
Ammonium sulfate precipitation	1,500	5,400	3.6	100	1
DEAE 5PW	75	3,804	50.7	70	14
Superdex S-200	40	3,180	79.5	59	22
Resource-Q	27	2,650	98.0	49	27

Molecular Masses

The apparent molecular mass of the native enzyme was estimated to be about 130 kDa by the gel filtration chromatography on Superdex S-200 as shown in Fig. 2A. The molecular mass of the C23O subunit was estimated to be 34 kDa after 12% Tricine/SDS-PAGE (Fig. 2B). These results are consistent with reported results that the enzyme consists of four identical subunits [17, 24]. The subunit structures of extradiol-type dioxygenases, which contain the ferrous form of iron, have been extensively studied [17, 24], and have been confirmed to be composed of four identical subunits. Crystalline metapyrocatechase was purified from an extract of *P. putida* mt-2, grown in a medium containing benzoate, by Nakai *et al.* [17]. They reported that the molecular weight of the preparation determined by the sedimentation equilibrium method was 140,000±3,000 daltons and the enzyme showed a single band on SDS-PAGE with 35,000 daltons. Purified 3-methylcatechol 2,3-dioxygenase from *P. putida* ATCC 23973 also showed a single band on SDS-PAGE corresponding to a subunit (molecular mass: 33,500±2,000 daltons) and the native molecular mass determined by gel filtration was found to be 120,000±20,000 daltons. This is consistent with the native enzyme being a tetramer of identical subunits [24]. Thus, bacterial catechol 2,3-dioxygenase is generally accepted to be composed of four identical subunits, which is known to have molecular masses of 33.5 kDa [17, 24]. In contrast, the protocatechuate 4,5-dioxygenase from *P. paucimobilis* consists of an equal number of two different subunits, α- and β-, of 18,000 and 34,000 daltons, respectively [18].

N-Terminal Amino Acid Sequence

Automated Edman degradation of the enzyme established the N-terminal sequence up to 30 residues (Fig. 3). The N-terminal amino acid sequence of C23O purified from *P. putida* SU10 was compared with those of extradiol dioxygenase of other bacteria [1, 2, 3, 6, 13, 16].

Several N-terminal amino acid sequences of extradiol dioxygenases were discussed previously [3]. However, among these extradiol dioxygenases, the enzymes which have C23O activities, such as *P. putida* mt-2 [16, 17], *P. putida* PpG7 [6], *Achromobacter xylosoxidans* KF707 [15], *P. putida* CF600 [1], and *P. putida* HS1 [2], are compared to *P. putida* SU10 in this paper. The alignment of the dioxygenase amino acid sequence with these other extradiol dioxygenases revealed a high identity, indicating that they are similar in primary structure. However, among the sequences of the enzymes from the six bacterial strains, positions 1, 16-19, 22, and 25-26 were not conserved. All of the C23O sequences begin with N-K-G, but the HS1 was different in that K is its first residue [2]. Instead of D-M-S-K at position 16-19, there are different amino acid residues at this position in PpG7 [6], and HS1 [2], which are especially different with N-L-E-A. The glutamic acid (E) at position 22 was found to be alanine (A) for CF600 and threonine (T) for the HS1 strain. In CF600 and HS1, the two amino acids at positions 25 and 26 are arginine (R) and aspartic acid (D) in contrast with SU10 which has valine (V) and glutamic acid (E) at the respective sites.

The results showed that N-terminal amino acid sequences (30 residues) of C23O exhibit high similarity even though C23O showed catalytic activities for catechol, 3-methylcatechol, and 4-chlorocatechol (as mentioned below). The C23O from HS1 and CF600 have small differences in the N-terminal amino acid sequence compared to the other four C23Os.

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                 5    10   15   20   25   30
SU10    NKGVMRPGHVQLRVLDMSKALEHYVELLGL
mt-2    NKGVMRPGHVQLRVLDMSKALEHYVELLGL
KF707   NKGVMRPGHVQLRVLDMSKALEHYVELLGL
PpG7    NKGVMRPGHVQLRVLDMGKALEHYVELLGL
CF600   NKGVMRPGHVQLRVLDMSKALAHYRDLLGL
HS1     KKGVMRPGHVQLRVLNLEAALTHYRDLLGL
        #              ####  #  ##
```

Fig. 3. Amino acid sequence alignment of the N-terminal domain (position 1 to 30) of extradiol dioxygenases from *Pseudomonas putida* SU10, *P. putida* mt-2 [16, 17], *Achromobacter xylosoxidans* KF707 [15], *P. putida* PpG7 [6], *P. putida* CF600 [1], and *P. putida* HS1 [2].
#; Nonidentical amino acid in all six sequences.

Substrate Specificity

As shown in Table 2, the catechol analogs such as 4-methylcatechol, 3-methylcatechol, and dihydroxybiphenyl were oxidized by C23O from *P. putida* SU10, but protocatechuic acid and homoprotocatechuic acid did not serve as a substrate. Even if the extradiol dioxygenases from *P. putida* can catalyze various substances, such as catechol, 3-methylcatechol and 4-methylcatechol [4, 22, 23, 24], it was assumed that C23O from *P. putida* SU10 is more specific for 4-methylcatechol than its analogs. Catechol and 3-methylcatechol were degraded at rates of 92% and 60%, respectively, compared with 4-methylcatechol.

Table 2. Effects of various substrates on the activities of C23O.

Substrate	Relative activity (%)
4-Methylcatechol	100
Catechol	92
3-Methylcatechol	60
Dihydroxybiphenyl	16
Homoprotocatechuate	0
Protocatechuate	0

The catechol 2,3-dioxygenase activity was measured spectrophotometrically in 50 mM phosphate buffer (pH 7.5) containing 0.5 mM substrate at 30±0.1°C.

Table 3. Kinetic parameters of C23O with different substrates.

Substrate	K_m (μM)	V_{max} (μM/min)	Relative K_{cat}/K_m
4-Methylcatechol	3.5	98.3	100
Catechol	4.4	83.6	67.6
3-Methylcatechol	4.6	43.0	33.1
Dihydroxybiphenyl	5.7	38.3	29.5

All values were determined from steady-state measurement at 30±0.1°C in 50 mM phosphate buffer, pH 7.5.

Dihydoxybiphenyl exhibits a lower binding affinity due to its bulky configuration of phenyl residue and thus the lower catalytic activity of the enzyme to dihydroxybiphenyl (Tables 2 and 3). When protocatechuic acid was incubated with the enzyme for 30 min, no change in the absorption spectrum of the compound was observed, indicating that procatechuic acid is not cleaved by C23O. This result seems to indicate that the affinity of the enzyme to the substrate was remarkably reduced due to the presence of a charge in the substrate molecules such as protocatechuate and homoprotocatechuate. The enzyme is thought to have high affinity to the non-charged substrates. These results were not in good agreement with the extradiol dioxygenase obtained from *Bacillus stearothermophilus* which reacts with homoprotocatechuate and protocatechuate [8]. The kinetics of catechol 2,3-dioxygenase for catechol, 4-methylcatechol, and 4-chlorocatechol by the determination of kinetic parameters will be discussed next.

Kinetic Calculations

The relationship between the enzyme activity and substrate concentration was the typical Michaelis-Menten curve. From this curve, Michaelis-Mentan constants such as K_m and V_{max} values for each substrate were determined by Lineweaver-Burk plots as shown in Table 3. The K_m and V_{max} for 4-methylcatechol were estimated to be 3.5 μM and 98.3 μM min^{-1}, respectively. The 4-methylcatechol and 3-methylcatechol which share similar conformation to catechol have K_m values of 3.5 μM and 4.6 μM, respectively, whereas catechol which has a similar affinity with 3-methylcatechol and dihydroxybiphenyl has somewhat lower affinity (K_m of 5.7 μM). The cleavage rate of catechol by the purified enzyme was compared with various extradiol-oxygenases from other sources [4, 23, 24]. The purified enzyme exhibited much higher affinity towards 4-methylcatechol than catechol and its analogs, but C23O also had a little higher catalytic activity than the other derivative catechols and a much higher catalytic activity on 3-methylcatechol (Table 3). These results were consistent with the data of substrate specificities as mentioned above.

However, the results are not consistent with other reported data [4, 15, 24], with each purified catechol 2,3-dioxygenase reacting with higher cleavage rates for 3-methylcatechol than catechol or 4-methylcatechol. Their results assumed that substitution at the 4-position of the catechol molecule decreased the affinity of the enzyme for the substrates. Their enzymes catalyzed the ring cleavage of catechol and 3-methylcatechol, but showed weak activity toward 4-methylcatechol.

The extradiol dioxygenase from *P. putida* SU10 has similar kinetics parameters as those purified from *P. putida* strains [4, 23, 24]. K_m and V_{max} values of the 3-methylcatechol were 4.6 μM and 43 μM per min, respectively, and those of the catechol were 4.4 μM and 83.6 μM per min, respectively (Table 3).

These data indicate that the conformation of 4-methylcatechol allowes it to preferentially bind to the enzyme compared to catechol and 3-methylcatechol. Moreover, catalytic activities for 4-methylcatechol are higher than those of 3-methylcatechol and catechol. However, no differences in the structural properties of the enzymes, such as the N-terminal sequence and molecular mass, were found.

Many *meta*-cleavage enzymes have been genetically and biochemically characterized [7]. The primary structures of the isofunctional enzymes found in various bacteria are different, suggesting that the catalytic properties of these enzymes may not necessarily be identical. In fact, catechol 2,3-dioxygenases from different bacteria exhibit different substrate specificities; C23O from *Pseudomonas putida* KT2440 exihibits preferred catalytic activity for catechol rather than for 3-methylcatechol or 4-methylcatechol [4], whereas the isofunctional enzyme from *P. putida* strain UCC2 or strain ATCC 23973 exhibits preferred activity for 3-methylcatechol rather than for catechol [24]. Such variability in the substrate specificity of isogenic enzymes may have resulted from natural selection imposed by the availability of initial substrates in the ecosystem, and from the chemical structures of intermediates, catechol or methylcatechol, produced from the initial substrates which were toluene, xylenes, and naphthalene. For further studies, determination of the whole amino acid sequence of the enzyme and the protein conformational structure by circular dichroism is necessary.

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