Characteristics of a Recombinant 2,3-Dihydroxybiphenyl 1,2-Dioxygenase from *Comamonas* sp. Expressed in *Escherichia coli*

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Abstract 2,3-Dihydroxybiphenyl 1,2-dioxygenase (2,3-DBDO) is an extradiol-type dioxygenase that involved in third step of biphenyl degradation pathway. The nucleotide sequence of the *bphC* gene from *Comamonas* sp. SMN4, which encodes 2,3-DBDO with His-tag, was cloned into a plasmid pQE30 in *E. coli*. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of the purified active 2,3-DBDO showed a single band around 33 kDa, corresponding the molecular mass of 2,3-DBDO subunit. Two fractions around 170 and 100 kDa were separated in gel filtration chromatography, but only former one (the fraction of 170 kDa) has 2,3-DBDO activity. The 2,3-DBDO was reported as the polymeric protein consisted of eight subunits. However, the fraction corresponding octameric protein of 2,3-DBDO was not found in the gel filtration chromatography. The 2,3-DBDO was exhibited the maximum activity at pH 9.0 and was stable at pH 8.0, relatively. The circular dichroism (CD) data showed that 2,3-DBDO had an α -helical folding structures at neutral pHs ranged from pH 4.5 to pH 9.0. However, this high stable folding structure was converted to unfolded structure in acidic region (pH 2.5) or in high pH (pH 12.0). The enzyme was thermally stable and active up to 40 °C. The conformational data by CD spectra were consistent with the stability of 2,3-DBDO by checking the activity. The

binding affinity (K_m) for 2,3-dihydroxybiphenyl, 3-methylcatechol, 4-methylcatechol and catechol was 11.7, 24 μ M, 50 mM and 625 μ M, respectively.

Keywords Dihydroxybiphenyl dioxygenase · *bphC* · *Comamonas* · Binding affinity · Circular dichroism spectra

Introduction

2,3-Dihydroxybiphenyl 1,2-dioxygenase (2,3-DBDO or BphC) has been found in various biphenyl-utilizing bacteria such as *Pseudomonas* sp. LB400 [1], *Pseudomonas pseudoalcaligenes* KF707 [2], *Pseudomonas putida* KF715 [3], *Pseudomonas* sp. KKS102 [4], *Comamonas testosteroni* B-356 [5] and *Rhodococcus* sp. RHA1 [6]. These bacteria catabolize the biphenyl to benzoic acid through the oxidative route. 2,3-DBDO catalyzes the extradiol ring cleavage of 2,3-dihydroxybiphenyl (2,3-DHBP) to 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (HOPDA) with the insertion of two atoms of oxygen [7]. This is one of the key reactions in the metabolism of the widespread pollutant like biphenyl.

In our laboratory, the microorganism having high degrading activity toward biphenyl from wastewater was isolated and identified as *Comamonas* sp. SMN4 [8]. The native 2,3-DBDO from *Comamonas* sp. SMN4 was purified using serial column chromatographies and its structural and the enzymatic properties were characterized [9]. The purified 2,3-DBDO from *Comamonas* sp. SMN4 was not characterized as an octameric homologous protein by the gel filtration chromatography [9]. This property was not identical with other 2,3-DBDOs: Other 2,3-DBDOs from *P. pseudoalcaligenes* KF707 [10], *P. putida* OU83 [11, 12] and *Pseudomonas* sp. KKS102 [4] were characterized as an

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octameric homopolymer. The gene cluster containing *bphC* gene encoding the enzymes on the catabolism of biphenyl has been also cloned from the genomic DNA of *Comamonas* sp. SMN4 and expressed them in *Escherichia coli* [13]. With the development of DNA recombinant techniques, the purification of interesting protein could be simplified by adding the specific affinity tags (e.g. His-tag) [14].

In this paper, the His-tagged recombinant 2,3-DBDO of *Comamonas* sp. SMN4 was expressed in *E. coli*, and the enzymatic characteristics for the recombinant 2,3-DBDO were investigated using the purified enzyme by Ni-NTA affinity chromatography. The enzyme properties of recombinant 2,3-DBDO were also compared with native 2,3-DBDO. Especially, it was investigated whether the polymeric protein (octamer) might be dissociated into monomeric protein by the column chromatography, or the native 2,3-DBDO from *Comamonas* sp. SMN4 consisted of monomeric protein.

Materials and Methods

Materials

Antibiotics, and aromatic compounds of biphenyl, catechol, 3-methylcatechol, and 4-methylcatechol were purchased from Aldrich Chemical Co. (St. Louis, MO, USA). 2,3-Dihydroxybiphenyl was obtained from Wako Chemicals (Osaka, Japan). Marker protein kit was purchased from Novagen (Madison, WI, USA). The bacterial strains used in this study were *E. coli* SG13009 (pREP4) (Qiagen, Hilden, Germany).

Construction of Recombinant Plasmid Expressing 2,3-DBDO

Most DNA manipulations were done as described by the methods of Sambrook et al. [15]. The region coding for the *Comamonas* sp. SMN4 *bphC* gene was PCR-amplified from a recombinant plasmid pNA210 [13]. The annealing temperature and other parameters were optimized to get specific amplification of *bphC* gene. The oligonucleotides used for PCR were chosen on the basis of the known DNA sequences of *bphC* gene (AY028943) as follows: oligonucleotide 1 (*Bam*H) (5'-CGCGGATCCCATGAGCATCGA-3') and oligonucleotide 2 (*Hind*III) (5'-TATAAGCTTGCCTGGCCGC-3'). The PCR products were digested with *Bam*HI and *Hind*III. A 879-bp DNA fragment (for *bphC*), containing the entire coding sequence, was isolated and cloned into the compatible sites of pQE30 (Qiagen). The His-tail, 13 amino acids (MRGSHHHHHTDP), added to N-terminal of the

protein. *E. coli* strain SG13009, harboring the recombinant pNCP plasmid, was grown for 3 h at 37 °C in Luria–Bertani (LB) medium (50 ml) containing 1 mM isopropyl- β -D-thio-galactopyranoside (IPTG; Takara, Shiga, Japan).

Purification of 2,3-DBDO

The cells harvested by centrifugation were suspended in 5 volumes (2 ml) of the sonication buffer (0.1 M Tris, 300 mM NaCl, 10 mM imidazole, pH 8.0) per gram of the wet cell paste, and the cells were disrupted by sonication. The cell lysate (approximately 1.5 ml) was centrifuged at $18,000\times g$ for 60 min, and the supernatant was mixed with 500 μ l of 50 % slurry of an nickel-nitrilotriacetic acid (Ni-NTA) resin suspension (Qiagen) in the sonication buffer. The mixture was agitated for 60 min at 4 °C and poured into an empty column (1 $\times$ 11 cm) to load on the resin. The resin was then washed in succession with 8 ml of washing buffer (0.1 M Tris, 300 mM NaCl, 20 mM imidazole, pH 8.0). The His-tagged proteins were eluted with the elution buffer (0.1 M Tris, 300 mM NaCl, 0.25 M imidazole, pH 8.0), and the fractions were collected and tested for the enzyme activity. After elution, Ni-NTA resin should be washed sequentially with 10 volumes of regeneration buffer (6 M guanidine hydrochloride, 0.2 M acetic acid) and stored in 30 % ethanol to inhibit the microbial growth.

Enzyme Assay

The 2,3-DBDO activity was assayed spectrophotometrically by measuring the formation of the *meta*-cleavage compound as described previously [9]. One unit of 2,3-DBDO activity is defined as the formation of 1 μ mol of *meta*-cleavage compound per minute at 25 °C.

Determination of Molecular Mass

The molecular mass of His-tagged 2,3-DBDO purified from *E. coli* was determined by gel filtration chromatography (Sephacrose CL6B; Pharmacia, Uppsala, Sweden) and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using the marker proteins as described previously [9].

pH and Temperature Effects and Kinetic Analysis on 2,3-DBDO Activity

The pH and temperature effects on 2,3-DBDO activity were measured with the same method described in previous paper [9]. The K_m and V_{max} values for 2,3-DBDO were determined with substrates 2,3-dihydroxybiphenyl, catechol, 3-methylcatechol and 4-methylcatechol as described

previously [9]. All measurements for kinetic studies including pH and temperature effect were carried out under the steady-state conditions with the substrates.

CD Spectra Determination

The conformation structure of each purified enzyme was determined by Jasco spectropolarimeter (Model 710, Hachioji, Japan) using 1 mm path-length cell. The salts in protein samples were removed by dialysis (MWCO, 5000) before CD work. The temperature was controlled by Jasco PTC-343 Peltier temperature controller. The protein concentration was adjusted approximately 5 μ M into water. The protein concentration was determined by BCA (Bicinchoninic acid) method [16]. The pH of solution was adjusted with 1 M NaOH and HCl at room temperature. The pH was measured before and after CD work for reconfirming the value [17]. For measuring temperature profile, the temperature of CD spectropolarimeter was programmed every 0.5 $^{\circ}$ C from 30 to 90 $^{\circ}$ C as follows: arising time, 1 $^{\circ}$ C min $^{-1}$; equilibrium time, 1 min; data collecting time, 1 min. All samples were degassed under the vacuum before CD experiments. All protein concentration was adjusted as 5 μ M. Molar ellipticity $[\theta]$ was calculated from the observed ellipticity (θ) in CD by following equation:

$$[\theta] = \frac{100 \theta}{C \ell}$$

where C is protein concentration ([M]), and ℓ is light path length (cm).

Results

Expression of 2,3-DBDO and Its Purification

His-tagged recombinant 2,3-dihydroxybiphenyl 1,2-dioxygenase (2,3-DBDO) from *Comamonas* sp. SMN4 was expressed well in *E. coli* SG13009 (pNCP). The cell broth containing expressed 2,3-DBDO turned yellow upon mixing with 2,3-dihydroxybiphenyl. About 1 g of cell paste was obtained from 50 ml culture of cell broth by centrifugation. The expressed protein was purified by Ni-NTA affinity chromatography from the cell paste. The yield of purified 2,3-DBDO was approximately 20 mg g $^{-1}$ of the cell paste (wet weight). Most of the unwanted proteins were washed out by 20 mM imidazole in the imidazole gradient purification system (Qiagen). The enzyme interested was eluted at 250 mM imidazole. No contaminating bands were detected on SDS-PAGE gels of His-tagged 2,3-DBDO prepared (Fig. 1a).

Determination of Molecular Weight

The molecular mass of monomeric protein for purified 2,3-DBDO estimated from the SDS-PAGE was to be approximately 33 kDa (Figs. 1a, 2a) which is corresponding to the molecular weight of the monomeric subunit of 2,3-DBDO. The molecular weight of expressed 2,3-DBDO calculated from amino acid sequences by the ExPASy molecular biology server including the 13 amino acid residues and the six His-tagged was 34,285.

At the same time, the molecular mass of the His-tagged 2,3-DBDO was estimated by gel filtration chromatography (GFC) to know whether the His-tagged 2,3-DBDO was polymeric protein (Fig. 1b). The GFC data for the crude extract of 2,3-DBDO expressed in *E. coli* showed two regions: Region 1 of 90–310 kDa with 2,3-DBDO activity and Region 2 of around 32 kDa without 2,3-DBDO activity (Fig. 1Ba). The molecular of Region 2 was corresponding to the monomeric subunit of native 2,3-DBDO. The peak corresponding to 280 kDa (second peak of Region 2) was very small (Fig. 1Ba). In case of the purified enzyme, two fractions were observed in around 170 kDa (Region 3) and around 100 kDa (Region 4) (Figs. 1Bb, 2b). Among the fractions, only the peak (around 170 kDa) showed 2,3-DBDO activity.

pH Effect on Expressed 2,3-DBDO

The expressed His-tagged 2,3-DBDO exhibited maximum activity at pH 9.0 and the activity declined in higher or lower pH regions than pH 9.0. Only 15 % of enzyme activity was retained even at neutral pH such as pH 6.5 (Fig. 3). The circular dichroism (CD) spectra for native and expressed 2,3-DBDOs at pH 8.0 which are determined by spectropolarimeter were shown in Fig. 4. The structural conformation of the expressed 2,3-DBDO was the almost same with that of the native enzyme. Both the expressed and native 2,3-DBDO showed a typical α -helical folding conformation at pH 8.0. They exhibited through around 210 nm and 222 nm, which were represented in typical α -helix structure [18].

The CD spectra for expressed 2,3-DBDO for the various pHs were shown in Fig. 5. The enzyme showed a typical α -helical folding structures at pHs ranged from pH 4.5 to pH 9.0. This structure maintained by pH 10.5, however, this high stable folding structure was converted to unfolded structure at high pH and low pH (Fig. 5). In pH 2.5, the structure of 2,3-DBDO was unfolded to random coil, whereas in high pH like as pH 12.0, the structure changed to unsimilar conformation. The CD spectra up to 215 nm from 250 nm showed helical like structure, but after 215–190 nm, the expressed 2,3-DBDO structure was highly distorted to random coil conformation (Fig. 5a)

Fig. 1 His-tagged 2,3-DBDO of *Comamonas* sp. SMN4 expressed from *E. coli* (a) and gel filtration chromatography (GFC) chromatograms for determining molecular mass of 2,3-DBDO (b). **A** SDS-PAGE was performed with 10 % concentration of SDS: lane 1 molecular marker standards; lane 2 crude preparation, lane 3 purified fraction obtained after elution of Ni-nitrilotriacetic acid resin. **B** GFC was performed using Sepharose CL6B for the crude extract (a), and enzyme purified after Ni-NTA affinity chromatography (b). The relative 2,3-DBDO activity is designated by closed circle

Fig. 2 Determination of molecular mass for the monomeric 2,3-DBDO from SDS-PAGE gel (circle) and the polymeric 2,3-DBDO by gel filtration (square). The marker proteins of the gel filtration used were: blue dextran (2000 kDa), ferritin (440 kDa), aldolase (158 kDa) and chymotrypsinogen A (25 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

Fig. 3 Effects of pH on activity (filled circle) and stability (open circle) of His tagged 2,3-DBDO expressed in *E. coli*. Citrate buffer for pH 4.0 and 5.0, sodium phosphate buffer for pH 6.0–8.0, Tris-HCl buffer for pH 7.5–9.0, and glycine buffer for pH 9.0–10.0 were used. Enzyme activities were determined by the initial velocity (v_0) under the standard conditions. For the stability test, enzyme was pre-incubated in each buffer at 4 °C for 24 h. The concentration of enzyme was $2.5 \mu\text{g ml}^{-1}$. The observed activities at the given pH were not the same between two buffers for the enzyme activity determination, but the relative activities at this pH were represented as a single point

[18]. It was very strange conformation that was not detected in any other usual proteins. The conformational structure changes were also observed in terms of pH by determining the ellipticity at 222 nm. Since at 222 nm of spectra, the maximum ellipticity change was detected between folding and unfolding structure [17], residual ellipticity at 222 nm was represented for comparing the

structure and activity of 2,3-DBDO (Fig. 5a). The CD spectra for the different pHs showed that the structure of enzyme was unfolded like as in random coil below pH 4.0, the structure was stabilized as increase of pH up to pH 6.0. After pH 6.0, the data of θ_{222} showed to level off as plateau even after pH 10.5 (Fig. 5B), in CD data of θ_{222} , the structure of enzyme look like stable after pH 10.5.

Fig. 4 CD spectra of 2,3-DBDOs for native (filled triangle) and expressed (open circle) in *E. coli* at pH 9.0

Fig. 5 CD spectra of purified His-tagged 2,3-DBDO for the various pHs (A). The inserted Figure (B) represented molar ellipticity of 2,3-DBDO at 222 nm ($[\theta]_{222}$) for the various pHs. Symbols: filled circle, pH 2.5; open circle, pH 4.5; filled triangle, pH 6.5; open triangle, pH 9.0; filled inverted triangle, pH 10.5; open inverted triangle, pH 12.0

However, the enzyme structure determined by CD was very unstable over pH 10.5 as shown in Fig. 5A. Thus, θ_{222} after pH 10.5 was not meaningful, anyway, the structure should be unfolded at high pH condition. The pH profile of enzyme structure was broader than the enzyme activity and enzyme stability profile (Figs. 3, 5). The enzyme after incubating 2,3-DBDO for 24 h at 4 °C for the different pHs was stable at neutral pHs although catalytic activity was very poor such a neutral pH as pH 7.5 (Fig. 6). In pH 9.0, at which 2,3-DBDO showed high activity, the enzyme lost their activity for 24 h incubation (Figs. 3, 5). Interestingly, however, the enzyme conformation (represented by α -helix) by CD was not changed for 24 h incubation at pH 9.0

Fig. 6 CD spectra of 2,3-DBDO for conformational stability at pH 9.0. For the conformational stability of enzyme, CD spectra of 2,3-DBDO for 24 h incubation at pH 9.0 (glycine buffer) for given periods were shown. Symbols: filled circle, 0 h; open circle, 8 h; filled triangle, 16 h; open triangle, 24 h

and 25 °C (Fig. 6), even though the enzyme lost their activity at optimum pH (pH 9.0) for long time incubation.

Temperature Effects

The temperature effects on 2,3-DBDO activity and the thermal stability were also determined at pH 9.0 by changing the temperatures from 10 to 100 °C. The activity increased as increase of reaction temperature up to 40 °C and 20 % of activity was reduced at 50 °C (Fig. 7a). The enzyme showed the maximal activity at 40 °C. The activation energy by Arrhenius plot was 3.78 kcal mol⁻¹ · K⁻¹. Up to 40 °C, the enzyme maintained its activity after incubating the enzyme for 24 h at the given temperatures (Fig. 7a). Figure 7c showed the spectra of 2,3-DBDO at the various temperatures. Up to 40 °C, the CD spectra were almost the same consistent with the stability data of 2,3-DBDO. After 60 °C, however, the CD spectra showed similar unfolding conformation which was shown at high pH conditions (Fig. 5). In the CD spectra on temperature effect, the wavelength where the maximum CD spectra changed between folding and unfolding structures was 208 nm instead of 222 nm (for pH effect, θ_{222} nm was chosen). Therefore, the change θ_{208} was for checking the temperature effect on CD. The mean residue ellipticity at 208 ($[\theta]_{208}$) showed that the conformation of 2,3-DBDO maintained their structure only up to 40 °C (Fig. 7b) which was consistent with the stability data of 2,3-DBDO enzyme (Fig. 7a).

Fig. 7 Effects of temperature on the enzyme activity (filled circle) and thermal stability (open circle) (A), CD spectra of the purified His tagged 2,3-DBDO expressed in *E. coli* (B), and ellipticity at 208 nm ($[\theta]_{208}$) plot versus temperature (C). **a** 2,3-DBDO activities were measured by determining the initial velocity (v_0) at given temperatures in 0.2 M glycine buffer (pH 9.0). Residual activities were measured under the standard condition at pH 9.0 and 25 °C. **B** CD spectra were observed by changing the temperatures after 2 min equilibrium. The concentration for CD work was the same as approximately 5 μ M. Symbols in C: filled circle, 10 °C; open circle, 20 °C; filled triangle, 40 °C; open triangle, 60 °C; filled inverted triangle, 80 °C; open inverted triangle, 100 °C. **C** Temperature stabilities were observed by determining the residual activities after incubating the enzyme for 24 h at each temperature in 0.2 M glycine buffer (pH 9.0)

Kinetic Calculations

Initial velocity (v_0) versus substrate concentration of 2,3-DBDO of *Comamonas* sp. SMN4 expressed in *E. coli* for four substrate analogues; 2,3-dihydroxybiphenyl, catechol, 3-methylcatechol, and 4-methylcatechol, showed the typical Michaelis–Menten curves. Kinetic parameters represented by Michaelis–Menten constants such as K_m and V_{max} values derived from Lineweaver–Burk plots were estimated to be 11.7 and 2700 μ M min^{−1}, respectively for 2,3-dihydroxybiphenyl (2,3-DHBP). The enzyme also bound to 3-methylcatechol, 4-methylcatechol, and catechol, and catalyzed the cleavage reaction with the different affinities expressed by the K_m values as 24 μ M, 50 mM, and 625 μ M, respectively (Table 1). The K_m values of this expressed 23DBDO were the same with those of the native enzyme from *Comamonas* sp. SMN4. In the case of the native 2,3-DBDO, the affinities expressed by the K_m values were 11 μ M, 25 μ M, 54 mM, and 615 μ M, for 2,3-

dihydroxybiphenyl, 3-methylcatechol, 4-methylcatechol, and catechol, respectively.

Secondly, 3-methylcatechol binds well to the 2,3-DBDO enzyme. However, 4-methylcatechol hardly binds to active site. No cleavage activity for 3-chlorocatechol, 2,3-dihydroxybenzoate, and 1,2-dihydroxynaphthalene was detected. Trace cleavage activity for 4-chlorocatechol by the expressed enzyme was observed (data not shown). The catalytic activity for 2,3-DHBP of 23DBDO was the most powerful as 2700 μ M min^{−1} of V_{max} . The V_{max} values of the enzymes for 3-methylcatechol, 4-methylcatechol, and catechol were 1740, 770, and 490 μ M min^{−1}, respectively. These V_{max} values of the expressed enzyme were similar to those of the native enzyme [9]. The substrate specificities expressed $V_{max} K_m^{-1}$ for 2,3-dihydroxybiphenyl, 3-methylcatechol, 4-methylcatechol and catechol, were 230, 72.5, 0.0154, and 0.784 min^{−1}, respectively (Table 1). In native 23DBDO, the $V_{max} K_m^{-1}$ values determined for 2,3-dihydroxybiphenyl, catechol, 3-methylcatechol, and 4-methylcatechol were 256, 0.167, 65.6, and 0.0120 min^{−1}, respectively [9]. These data meant that the enzyme 2,3-DBDO for *Comamonas* sp. SMN4 having higher catalytic activity for 2,3-dihydroxybiphenyl catalyzed 3-methylcatechol in substantial. But, for 4-methylcatechol and catechol, the enzyme had very poor activity.

Discussion

Furukawa group and other investigators reported that 2,3-dihydroxybiphenyl 1,2-dioxygenase from various microorganisms such as *P. pseudoalcaligenes* KF707 [19], *Pseudomonas* sp. LB400 [10] and *P. putida* OU83 [11] consisted of eight subunits. Usually, the polymeric protein composed of subunits was not dissociated during the gel filtration [20]. Thus, the molecular mass of 2,3-DBDO on the gel filtration chromatography (GFC) might be the around 270 kDa if the 2,3-DBDO consisted of eight identical subunits as reported as other investigators. However, our data of GFC did not show that the 2,3-DBDO expressed in *E. coli* was the octameric protein even in the crude extract. The peak corresponding to 280–300 kDa (octameric molecular mass of 2,3-DBDO) was very small (Fig. 1Ba). Furthermore, the peak was not found in the purified enzyme.

This result was consistent with the data of native 2,3-DBDO from *Comamonas* sp. SMN4. In previous report [9], we did not found any direct evidence on the gel filtration chromatography that the 2,3-DBDO enzyme from *Comamonas* sp. SMN4 was the polymeric protein composed of eight identical subunits (32 kDa). In native 2,3-DBDO, the 2,3-DBDO activity was observed in the monomeric fraction for the purified enzyme [9]. However, in the expressed

Table 1 Kinetic parameters of 2,3-dihydroxybiphenyl 1,2-dioxygenase of *Comamonas* sp. SMN4 which was expressed in *E. coli* with different substrates

Compounds	K_m (μM)	V_{max} ($\mu\text{M min}^{-1}$)	$V_{max} K_m^{-1}$ (min^{-1})
2,3-Dihydroxybiphenyl	$11.7^a \pm 0.7$	2700 ± 85	230
3-Methylcatechol	24 ± 2.3	1740 ± 60	72.5
4-Methylcatechol	$50,000 \pm 450$	770 ± 35	0.0154
Catechol	625 ± 12.5	490 ± 19	0.784

All values were calculated from the Lineweaver–Burk plot of Michaelis–Menten curve which was observed by determining the initial velocity from steady-state measurement at 25 °C in 0.1 M glycine buffer pH9.0. K_m and V_{max} are indicated as mean $\pm$ SD for five determinations

enzyme, the fraction corresponding to monomeric protein (subunit; 33 kDa) was not found even in the purified enzyme. The 2,3-DBDO activity was observed only in the Region 1 [in crude extract (Fig. 1Ba)] and Region 3 [in the purified 2,3-DBDO (Fig. 1Bb)]. The molecular weight of Region 3 was around 170,000, which might be the molecular weight of a pentameric polymer of 2,3-DBDO subunit. Region 4 in Fig. 1Bb was deducted as trimeric polymer of the 2,3-DBDO subunit.

In fact, the gel filtration for the standard proteins (urease (tetramer), urease (dimer), and alcohol dehydrogenases) with this column showed that the polymeric protein was not dissociated. Additionally, Cao et al. [21] reported that three recombinant 2,3-DBDO expressed in *E. coli* were a homotetramer or homodimer protein and all of them possessed high 2,3-DBDO activity. Thus, it represents that the 2,3-DBDO proteins (native and recombinant protein) in our study may be a monomeric protein, but also pentameric or octameric protein. However, it was still questionable that the purified 2,3-DBDO expressed in *E. coli* was the pentameric protein or why the 2,3-DBDO dissociated into pentamer and trimer. Any data was not found that the purified 2,3-DBDO in *Comamonas* sp. SMN4 or expressed in *E. coli* was the octameric protein.

In addition, it is not certain why the gel filtration chromatograms for native and expressed 2,3-DBDO were different: The native 2,3-DBDO was observed as the monomeric protein, while the expressed 2,3-DBDO was the pentameric protein. Only the difference between the

native and expressed 2,3-DBDO was the His-tail (MRGSHHHHHTDP) in N-terminal of 2,3-DBDO, and expression system. Although His-tags have gained great popularity over the last decade as a purification tool for recombinant proteins, sometimes but often tagged 6-His chains made the problem to disturb the native structure of protein and rarely His-tag reduced some enzyme activities [22]. For this reason, 2,3-DBDO activity in recombinant monomeric or trimeric protein (Region 2 in Fig. 1Ba and Region 4 in Fig. 1Bb) may not have been detected. Most of extradiol dioxygenase monomers consist of a five-coordinate square pyramidal Fe^{2+} center in active site, with three His, one Tyr and one Glu residue. The amino acids also require enough time and space to coordinate the Fe^{2+} responsible for catalytic role. But if the active site was not completely developed and may not facilitate the access of the substrate to the active site of the enzyme, 2,3-DBDO activity would not be observed. It could be another reason for inactivation of recombinant 2,3-DBDO.

The maximum activity of the 2,3-DBDO expressed in *E. coli* was observed at pH 9.0, while the enzyme was maintained its stability at pH 7.5 during the 24 h incubation. In native 2,3-DBDO, the enzyme was stable at pH 8.0 [13], the native enzymes are also not most stable at pH where the 2,3-DBDO shows the maximum activity (pH 9.0). In previous paper [9], we suggested that the phenomena were due to structural difference between the active site and whole protein. In binding Fe^{2+} in the coordination system, only negative charged form

(deprotonated) in *etha* nitrogen (N^{ϵ}) of imidazole ring (His) was capable to make a coordination system. Theoretically, below the pH 6.8, N^{ϵ} atom was protonated and lost the ability to coordinate to the Fe^{2+} . This means that totally deprotonated negative charge of two His residues were advantageous to make the coordination system for the activity. Thus, optimal pH of the 2,3-DBDO must be much higher than 6.8, like as pH 9.0 for *P. pseudoalcaligenes* KF707 [19] and pH 8.5–9.5 for *Rhodococcus* sp. strain DFA3 [23] and pH 8.0 for *Pseudomonas* sp. LB400 [10] and *P. putida* OU83 [11]. That is reason why the 2,3-DBDO of *Comamonas* sp. SMN4 showed the highest activity around at pH 9.0.

The protein structures were not well maintained at the basic conditions such as pH 9.0 or higher pH with exception of some enzyme. In the Fig. 3, the 2,3-DBDO protein was stable in the neutral pH (pH 7.5). It was expected, microscopically around the active site, pH 9.0 was highly effective to make coordination system for the catalytic reaction of the 2,3-DBDO, but whole protein structure of the 2,3-DBDO enzyme was not stable at pH 9.0. When 2,3-DBDO was incubated for long time at pH 9.0, the enzyme lost its catalytic activity (Fig. 3).

The tertiary structure might be corrupted at pH 9.0, thus, the enzyme was unstable and lost the enzyme activity. However, the conformation of the 2,3-DBDO structure determined by CD was maintained even at pH 9.0 for 24 h (Fig. 6), meaning that the enzyme at pH 9.0 was not totally denatured. In fact, the conformational structure (secondary structure determined by CD) was highly conserved in case of molten globule state [24], although the enzyme lost its catalytic activity. However, at extreme pH, the enzyme should be totally denatured (Fig. 5a).

As shown in Fig. 7, although the 2,3-DBDO enzyme expressed in *E. coli* was very sensitive to the temperature especially at over 40 °C, the enzyme was stable for 24 h incubation up to 40 °C. The thermal properties of expressed 2,3-DBDO were almost the same with those of native 2,3-DBDO. For the case of native 2,3-DBDO enzyme from *Comamonas* sp. SMN4, the 2,3-DBDO was stable up to 30 °C for 24 h incubation and the enzyme showed maximal activity at 40 °C [9].

The expressed 2,3-DBDO have the same binding affinity (K_m^{-1}) and the catalytic activity (V_{max}) for the 2,3-dihydroxybiphenyl (2,3-DHBP), 3-methylcatechol, 4-methylcatechol and catechol as the native enzyme (Table 1). The side residues at 3 (*ortho*) position of catechol ring were benzyl in 2,3-DHBP, methyl in 3-methylcatechol, and hydrogen in catechol. In 4-methylcatechol, methyl group was bound 4 (*para*) position, instead of 3 (*ortho*) position of catechol. As mentioned above, deprotonated imidazole rings of two His in active site were involved to make a coordination system. The substrate, 2,3-DHBP binds with

the help of Tyr and His, two hydroxyl groups of catechol ring are oriented to Fe^{2+} ion. The residues of benzyl ring in 2,3-DHBP, methyl in 3-methylcatechol, and hydrogen in catechol are oriented to the direction of outer Tyr.

For the 2,3-DHBP, the side chains of 2,3-DHBP (benzyl) oriented to the phenyl group of Tyr and probably phenyl group of Tyr helps the 2,3-DHBP binding by hydrophobic interaction or supporting the stable orientation of substrate to Fe^{2+} complex. Stable and correct orientation of substrate to active site is essential for the reaction.

The data (Table 1) showed that 3-methylcatechol could bind to this Fe^{2+} ion coordination system with the same orientation of 2,3-DHBP. The side chain (methyl) of 3-methylcatechol is much shorter and smaller than that of 2,3-DHBP (benzyl). Thus, in the active site of 2,3-DBDO enzyme, the methyl group of 3-methylcatechol could not make a correct proximity but proper orientation with phenyl group of Tyr residue, because the methyl group is shorter than phenyl group. Phenyl group of Tyr cannot have high affinity with 3-methyl group of 3-methylcatechol.

Without the help of side chain such as benzyl and methyl groups, the catechol could bind to the Fe^{2+} ion coordination system with very low affinity. Also, the bound 3-methylcatechol and catechol should be less stable in the active center [4]. Therefore, the rate of catalytic reaction was less active than its 2,3-DHBP. The fact 3-methylcatechol is more active than catechol by 3 ~ 4 times means that 3-methylcatechol binds to the active site of enzyme more stably than catechol.

For catalytic reaction in 4-methylcatechol, 4-methyl group in catechol should be oriented to imidazole ring of His instead of phenyl group of Tyr. Because His residue was located at opposite site of Fe^{2+} , the binding of 4-methyl group of 4-methylcatechol was inhibited by molecular hinderance of His residue with limited space. As stated above, this might be a reason only 4-methylcatechol rarely bound to the active site of the enzyme for the catalytic reaction (K_m of 50 mM). However, the enzyme had catalytic capability to the bound 4-methylcatechol with 770 ($\mu\text{m min}^{-1}$) of V_{max} which was greater than catechol, meaning that extradiol ring cleavage in 4-methylcatechol if bound took place [9]. The substrate specificity of enzyme depends on the correct orientation of bound substrate, the proximity between substrates and Fe^{2+} coordination, and the stability of a enzyme substrate complex. Detailed structural analysis of the enzyme reaction mechanism will be studied by preparing one point mutant protein by site-directed mutagenesis.

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