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토양 박테리아로부터의 Pyrocatechase에 관한 연구(제1보). 효소정제와 특성연구

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Studies on Pyrocatechase from a Soil Bacterium (I). Purification and Characterization of Pyrocatechase

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요 약. 토양에서 분리한 Pseudomonadaceae 속 박테리아로부터 pyrocatechase를 추출, 분리 정제하였으며, 이 효소의 특성을 검토한 결과 pyrocatechase는 catechol에 대하여 기질 특이성을 보여줌을 알았다. 효소 활성도의 최적조건은 pH 7~10 부근과 온도 35 °C 임을 알았으며, catechol에 대한 K_m 값은 $1.9\times10^{-6}M$로 얻어졌다. 기질 유도체에 의한 효소 저해 실험결과 벤젠 고리의 *ortho* 위치에 두개의 수산기는 효소-기질간의 결합반응에 참여될 것이라고 추측했다. 기타 SH-잔기와 작용하는 화합물 또는 중금속 이온등의 첨가에 따른 효소 활성도의 저해 효과를 검토 하였으며, 효소 활성부위에 대하여도 검토해 보았다.

ABSTRACT. Pyrocatechase as a phenolytic dioxygenase was extracted from the benzoate-induced cells of a soil bacterium, a member of *Pseudomonadaceae*, and purified partially by DEAE-cellulose ion-exchange chromatography and Sephadex G-75 gel filtration. Final preparation of the enzyme yielding 200 fold purification over the crude extracts showed a specific activity of about 40 μmoles per minute per mg protein based on catechol as the substrate. The enzyme showed a very limitted substrate specificity towards catechol for its catalytic activity. Based on the inhibition study with the substrate analogues, it was assumed that *ortho* dihydroxy groups on the aromatic ring may participate in the enzyme-substrate binding. The K_m value for catechol was obtained as 1.9×10^{-6} M, and the optimum activity of the enzyme was obtained at the pH range of 7~10 and 35°C. With SH-group blocking agents the enzyme was inhibited seriously. The activity of enzyme was also inhibited by the addition of some heavy metals, Ag^{+} and Cu^{2+}, but was not affected by EDTA. General property of the enzyme was characterized and the possible nature of the enzyme active center was also discussed.

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INTRODUCTION

Pyrocatechasc (Catechol: Oxygen 1, 2-oxidoreductase, EC 1. 13. 11. 1) as a typical phenolytic dioxygenase has been known to cleave the aromatic ring of catechol to *cis, cis*-muconic acid with the consumption of two atoms of the molecular oxygen[1~3]. The phenomenon of biological oxygen fixation by a new class of oxidoreductive enzymes, oxygenases, originally demonstrated by two independent groups of Hayaishi[4] and Mason[5] in 1955, and then later the nature of phenolytic dioxygenases as a subclass of oxygenases was also established by Hayaishi *et al.* suggesting that they may have an important role in the β-keto adipic acid pathway or in the other catabolic metabolism of aromatic compounds in the living organisms[7,8].

The enzymes capabling the molecular oxygen fixation to a variety of metabolites are known to occur widely in nature, from bacteria to human[9,10], but phenolytic dioxygenases specific to the aromatic compounds are found mainly in the microbial origin[11,13]. Since there are also a quantity of aromatic compounds distributed widely in our environment, it has been much interested during the past three decades in the study on microbial dioxygenases as well as on the screening of the related microorganisms which participate in the oxidative degradation of certain aromatic compounds.

The naturally occuring aromatic compounds are generally known to be water insoluble due to their oxygen-deficient nature, and thus most of them are biologically inactive, or in some case, they are very toxic to the living matters. To become the more water soluble and bioactive components, they may need to be oxygenated by aromatic compound-specific microbial dioxygenases including pyrocatechase. Therefore, the study on pyrocatechase from several different bacteria has recently been paid much attention in the aspects of its ecological, pharmacological and physiological view points.

Although there have been many fractional suggestions put forwards the nature and specific function of microbial dioxygenases, there is no comprehensive view yet on the molecular property of pyrocatechase. In the present communication, a new bacterium of Pseudomonad family was screened from soil samples, and from that screened microorganism pyrocatechase was fractionated and purified partially. The functional and molecular properties of the enzyme were characterized and discussed with the results from others.

MATERIALS AND METHOD

Chemicals. Catechol, resorcinol, hydroquinone, and gallic acid were the products of Wako Pure Chem. Ind. Co., Japan. Protocatechuic acid, 4-nitrocatechol, N-ethylmaleimid(NEM), *p*-chloromercuribenzoate(PCMB), and iodoacetamide were purchased from Sigma Chem. Co., U. S. A. Pyrogallol was obtained from Aldrich Chem., Germany. Tiron(4, 5-dihydroxy *m*-benzene difulfinic acid disodium salt), and sodium dithionite were from Merck, Germany. DEAE (diethylaminoethyl)-cellulose ion exchanger was obtained from Bio-Rad Lab., U. S. A. while Sephadex G-75 was from Pharmacia Fine Chem., Sweden. *o*-Phenanthroline was the product of Fisher, U. S. A., and α, α'-dipyridyl was imported from Kanto Chem. Co., Japan. All other chemicals used were of the analytical grade from commercial sources.

Screening of a soil bacterium. A bacterial strain capable to degradate aromatic compounds was screened from soil samples by the method of Hayaishi[14], and a homogeneous colonies was selected through repeated transfer for its rapid growth under the enrichment culture medium

containg sodium benzoate as a sole carbon source. The tests for the identification of the microorganism were followed by the Bergey's Manual[15] and the modified method of Harrigan and McCance[16]. To confirm the homogeneity of the microorganism Gram's stain was also applied.

Cell Growth. The screened microorganism was cultured for 2 days at 25 °C in a 4 liter fermentor under the culture medium containg sodium benzoate, 3 g/*l*; yeast extract, 1 g/*l*; amonium phosphate, 3 g/*l*; K_2HPO_4, 1.2 g/*l*; KH_2PO_4, 0.5 g/*l*; and $MgSO_4 \cdot 7H_2O$, 0.2 g/*l*. The cell growth rate was detected by measuring the increase in the optical density at 540 nm[17]. The culture was entered stationary phase after 40 hours growth, and at that period approximately 15 g of wet cells were obtained from 4 liters of the medium. The cell pellets thus obtained were washed with 0.85 % NaCl solution, and stored at −10 °C.

Assay of Pyrocatechase Activity. The activity of pyrocatechase was measured spectrophotometrically the amounts of muconic acid produced from catechol as a substrate. A Varian Techtron Model 635 *M* UV-VIS recording spectrophotometer was used to measure the increase in the absorbance of muconic acid at 260 nm ($A_{260}=1.5\times10^4$)[18]. The standard assay system contained 0.2 μmole of catechol, 100 μmoles of *Tris*-HCl buffer, pH 8.5, and 0.1∼0.5 m*l* of the enzyme preparation in a total volume of 2.0 m*l*. The rate of the enzyme reaction was recorded continuously for 5 minutes, and the initial velocity was used as the enzyme unit. One unit of the enzyme activity was defined as 1.0 μmole of muconic acid formation of catechol decomposition per minute[18]. As pyrocatechase yields muconic acid derivatives by cleaving the aromatic ring, the substrate specificity experiments for the enzyme could be easily performed by measuring the absorbance at 260 nm due to the delocalizing double bonds of catechol analogues. The products of the enzyme reaction with several substrate analogues were analyzed by thin-layer chromatography. The protein concentrations of the enzyme preparation were measured by Lowry method[19], but for routin chromatographic experiments, the relative A_{280} was measured by ISCO Model UA-5 monitor.

Enzyme Extraction and Purification. Each 10 g of cell pellets was suspended in 40 m*l* of 0.1 *M* *Tris*-HCl buffer, pH 8.0 at 4 °C for ultrasonic disintegration. Sonic oscillation was performed for 15 minutes at 70 % of the full power with Sonic 300 Dismenbrator from Artex System Co. After centrifugation of the sonicated cells at 10,000×g for 30 minutes, the precipitate residue was discarded, and the supernant solution as cell-free extracts were fractionated by ammonium sulfate at the concentration range between 20∼50 % saturation. The resulting ammonium sulfate cake was dissolved in 20 m*l* of 0.01 *M* *Tris* buffer, pH 8.0, and dialyzed against the same buffer for 20 hours. The dialysismembrane, Union Carbide cellophane membrane, was activated before use according to McPhie[20]. The dialyzed enzyme preparation obtained after centrifugation showed only 2.5 fold purification. The further purification of the enzyme was carried out with DEAE-cellulose ion-exchange resin and followed by Sephadex G-75 gel filtration. A column of DEAE-cellulose resin, 2.2×30 cm, was previously equibrated with 0.05 *M* *Tris*-HCl buffer, pH 8.5, and 75 m*l* of the enzyme sample was loaded. After subsequent elution with the same buffer, a linear salt gradient with 0.5 *M* NaCl was established. The column flow rate was adjusted to 20 m*l* per hour, and 3 m*l* fractions were collected by ISCO fraction collector equipted with UA-5 flow cell monitor. About 7 fractions showing high

enzyme activity were collected and applied susequently to a Sephadex G-25 gel column, 2.5×15 cm for desalting. As the final step of purification, the above desalinated enzyme sample was applied to a Sphadex G-75 gel column, 2.5×60 cm, and eluted with the same *Tris*-HCl buffer, pH 8.5, at the flow rate of 20 m*l* per hour. In this case, most of proteins appeared in the void volume range, but the highest enzyme activity was founded at the shoulder of protein peak. The final preparation collected from the gel filtration yielded only 12 % of the original enzyme, but showed about 200 fold purification over the crude extracts. The homogeneity of the enzyme preparation was examined by disc gel electrophoresis on poly acrylamide gel columns according to the method of Brewer and Ashworth.

The Enzyme Characterization Studies. The enzyme assay for studies on the enzyme characterization was carried out under the standard assay condition if not otherwise mentioned. The substrate, catechol and its analogues are found to be very labile to autooxidation by air or light so that their stock solutions were protected from the light and air by storing them at 4 °C in the screw capped test tubes wrapped with aluminium foil. The buffer systems used for the pH-dependent experiments are 0.05 *M* buffers of sodium acetate for pH below 6, sodium phosphate for pH 6~8, *Tris*-HCl for pH 8~9.5, and diethanolamine-HCl for the pH ranges higher than 9.5. When the enzyme assay was undertaken under the alkaline condition, 0.5 m*M* of glutathion (GSH) was added to prevent the autoxidation of catechol.

RESULTS

Characterization of the Screened Microorganism. The identification and characterization of the screened microorganism from soil samples were carried out by general routin method, and the results are shown in *Table* 1. The color of the colony was pale brown. The bacterial cells were sticky but smooth, and showed fast vibrational motility at hanging drop test indicating the presence of flagella. Therefore, it was concluded that our new bacteria from soil samples might be a member of Pseudomonadaceae.

Cell Growth and the Enzyme Purification.

Table 1. Properties of the screened microorganism for pyrocatechase.

Test*	Method**	Result
1. Phototrophy	Incubation in dark	(+)
2. Filament & Sheath	Microscopy	(−)
3. Fission	Microscopy	Identical cells
4. Cell wall	Microscopy	Rigid cell wall
5. Gram's stain	Microscopy	(−)
6. Parasitism	Culture	(−)
7. Rod or coccus	Culture	Bacilli
8. Aerobiosis	Stab culture	(+)
9. Motility	Hanging-drop method	(+)
10. Dimension of cell	Microscopy	1×3.5 μm
11. Colony	Slant culture	White, convex, circular, entire

* The tests were applied according to the key in the Bergey's Manual (15).
** The methods for the test were adapted from Harrigan *et al.* (16).

The cell culture entered the stationary phase after 40 hour growth period, and when the cells were harvested at that period, about 15 g of wet cells was obtained from 4 liters of the culture medium. The optimum culture condition was found to be at 25 °C and at neutral pH. The enzyme, pyrocatechase, seemed to be inducible since the activity of the enzyme per unit cell was increased about 10-fold under the culture medium containg sodium benzoate instead of glucose.

The cell-free enzyme extracts obtained by ultrasonic treatment showed a specific activity of 0.25 units per mg protein, but after purification of the enzyme by DEAE-cellulose ion-exchange column and Sephadex G-75 gel filtration, the specific activity of the enzyme was increased about 200-fold. The result of the enzyme purification was summerized in *Table* 2. The final preparation of the enzyme was found to be relatively homogeneous based on polyacrylamide disc gel electrophoresis although the major enzyme fractions from a Sephadex column were collected at the shoulder region of the protein peak.

General Characteristics of Pyrocatechase from a Soil Bacterium. The molecular weight of the enzyme was estimated to be about 7×10^4 based on Sephadex G-75 gel filtration, but it was not rechecked by any further experiment. The enzyme, pyrocatechase, from a soil bacterium seemed to have a strict substrate specificity towards catechol. As shown in *Table* 3, among the several phenol derivatives serving as the substrates for the known phenolytic dioxygenases from different microorganisms, only catechol was degradated oxidatively by our enzyme preparation, and the product of the enzyme reaction was identified as *cis*, *cis*-muconic acid by thin-layer chromatography and by its characteristic UV-absorption spectrum. The enzyme preparation was found to have only the activity of pyrocatechase, but no activity of metapyrocatechase producing β-hydroxy muconic acid semialdehyde *via meta*-fission of catechol was detected. The enzyme was found to be stable relatively at room temperature and even at the alkaline condition of pH 9.0 for several days. As shown in *Fig.* 1, the optimum activity of the enzyme was obtained at the broad pH range of 6.5 to 10.5. Under the alkaline condition catechol and its derivatives were also found to undergo some degree of autooxidation, but by the introduction of glutathion to the enzyme reaction mixture, the rate of the enzyme catalysis was not much affected by the substrate autolysis. The optimum temperature for the enzyme was found to be at the range of 35° to 40°, and the activation energy of 12.6 kcal per mole was estimated froman Arrhenius plot. The

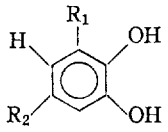
Table 2. Purification of pyrocatechase from a soil bacterium.

Procedure	Volume (ml)	Enzyme* activity (units/ml)	Protein** conc. (mg/ml)	Specific activity (unit/mg)	Purification (fold)	Yield (%)
Crude Enzyme	200	3.78	18.4	0.45	1.0	100
$(NH_4)_2SO_4$ Fractionation (20~55%)	75	3.85	7.4	0.52	2.5	38
DEAE-Cellulose Chromatography	20	10.35	0.8	12.5	59.5	27
Sephadex G-75 Chromagraphy	6	17.17	0.4	42.9	204.4	12

* The enzyme activity was measured by spectrophotometry at 260 nm.

** Protein concentration was determined by the method of Lowry *et al.* (19).

Table 3. Substrate specificity of pyrocatechase from a soil bactium.



Aromatic polyol	Substituent group		Relative activity* (%)
	R_1	R_2	
Catechol	H	H	100
4-Nitro catechol	H	NO_2	0
Pyrogallol	OH	H	0
Protocatechuic acid	H	COOH	0
Gallic acid	OH	COOH	0

* The enzyme activity was measured spectrophotometrically at 260 nm. The concentration of tested compounds was 1 m*M*.

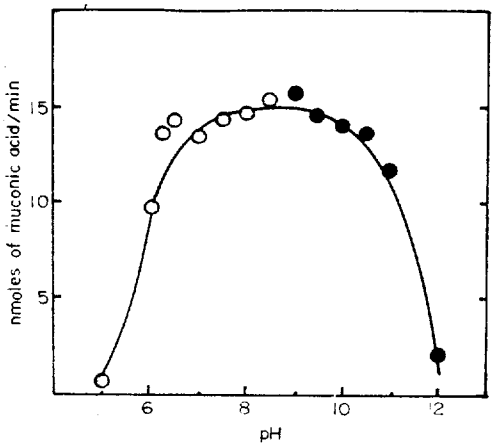


Fig. 1. pH-rate profile of pyrocatechase from a soil bacterium.
The enzyme activity was assayed under the standard condition at various pH range using different buffers (0.05*M*). Acetate buffer was used for pH below 6; Na-phosphate buffer, pH 6 to 7.5; *Tris*/HCl buffer, pH up to 9.5; diethanolamine/HCl buffer, pH above 9.5. When the enzyme activity was measured at alkaline condition, pH above 8.5, 0.5 m*M* of glutathion was added to the reaction mixture (indicated by filled circles). The buffer concentration was 0.05 *M*.

variation of the enzyme activities on the concentrations of catechol showed a typical hyperbolic nature of the Michaelis-Menten type, and

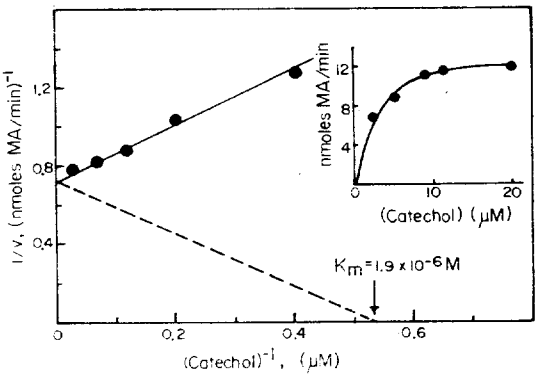


Fig. 2. Concentration dependent pyrocatechase activity with catechol.
The enzyme activity was assayed under the standard condition at pH 8.5 with varying concentrations of catechol as the substrate.

Table 4. Effect of substrate analogues on the activity of pyrocatechase from a soil bacterium

Substrate analogue added	Concentration (*M*)	Activity* (units/mg)	Relative activity
Control	—	0.542	100
Phenol	1×10^{-4}	0.540	99.6
p-Hydroquinone	1×10^{-4}	0.291	53.9
Resorcinol	1×10^{-4}	0.564	104.4
Protocatechuic acid	1×10^{-4}	0.528	97.8
4-Nitro catechol	1×10^{-4}	0.096	17.7
Pyrogallol	1×10^{-4}	0.147	27.2
Gallic acid	1×10^{-4}	0.549	85.0

* The enzyme activity was assayed under the standard condition as described in the text.

the affinity constant (K_m) of $1.9\times10^{-6}M$ for catechol was estimated from a Lineweaver-Burk plot as shown in *Fig.* 2.

Inhibition by Substrate Analogues. Effect of substrate analogues on the pyrocatechase-catalyzed catechol oxidation was studied with several aromatic polyols for an information on the nature of the enzyme-substrate binding. Among the several aromatic alcohols tested, the only compounds having the *ortho*- or *para*-dihydroxy group, *i.e.*, hydroquinone and pyrogallol, were found to inhibit the enzyme significantly

as shown in *Table* 4, but no inhibition was observed with phenol and resorcinol. In addition, it was also noticed that 4-nitro-catechol exhibits the greatest inhibition among the tested analogues while protocatechnic acid having 4-carboxyl group instead of nitro group on the catechol ring had no effect on the enzyme activity. By the introduction of the carboxyl group on the pyrogallol ring, the inhibitory effect of pyrogallol was reduced remarkably. Therefore, it can be speculated that the *ortho-* or *para-* dihydroxyl group on the aromatic ring may participate in the enzyme-substrate binding and that affinity of such dihydroxy group to the enzyme may also be enforced by the presence of an uncharged electron withdrawing group like a nitro group.

Effects of Metal Ions and Functional Group Blocking Agents. In the presence of some heavy metal ions such as Ag^+ and Cu^{2+}, the enzyme activity was inhibited remarkably as shown in *Table* 5, however by the addition of 1.0 m*M* EDTA to the metal-inhibited enzyme reaction system, the full enzyme activity was restored. Effect of several metal-chelating agents on the enzyme was also demonstrated by the preincubation experiments since the enzyme has been thought as a melloprotein having a non-heme iron as a prosthetic group. Among the tested metal chelators, *o*-phenanthroline and Tiron which are known to react specifically with di- and trivalent metal ions were found to give a marked inhibition while EDTA did not affect on the enzyme activity at all (*Table* 5). By the addition of arsenite acting on the viscinal thiol groups, the enzyme activity was also inhibited to certain extent, but the more marked inhibition was obtained with *p*-chloromercuric benzoate (PCMB) and *Bis*-dithio-(2-nitro benzoic acid) (DTNB). Among the alkylating agents tested, N-ethyl maleimide (NEM) showed a strong inhibition while iodoacetate (IAA) did not much affected on the enzyme activity.

Table 5. Inhibition of the pyrocatechase activity by heavy metal ions and metal chelating agents.

Compound added	Concentration (m*M*)	Activity (units/mg)	Percent inhibition (%)
Control	—	0.542	—
Silver nitrate	0.1	0.000	100.0
Cupric sulfate	0.1	0.110	61.1
Sodium arsenite	1.0	0.208	61.6
$FE(NH_4)_2(SO_4)_2$	0.1	0.430	20.7
$FE(NH_4)(SO_4)_2$	0.1	0.486	10.4
EDTA	1.0	0.530	2.0
α, α'-dipyridyl	0.1	0.518	4.4
o-Phenanthroline	0.1	0.500	7.5
Tiron	0.1	0.270	50.0
PCMB	0.1	0.102	81.1
DTNB	0.1	0.306	43.5
N-Ethylmaleimide	0.1	0.054	90.0
Iodoacetamide	0.1	0.464	14.8
Cystein	0.1	0.545	No Inhibition

* The enzyme activity was assayed under the standard condition as described in the text. PCMB, *p*-chloro mercuribenzoate; DTNB, 5, 5′-dithio-*bis*-(2-nitrobenzoic acid); Tiron, 4, 5-dihydroxy *m*-benzene sulfinic acid disodium salt.

DISCUSSION

The extract of benzoate-induced culture of a screened soil bacterium, Pseudomonadeceae was found to contain the activity of pyrocatechase catalyzing an oxygenative intradiol cleavage of catechol to *cis, cis*-muconic acid, and the purified enzyme having the molecular weight of about 7×10^4 showed a red color with broad absorption between 380 and 600 nm. The absorption peak of this enzyme preparation at 440 nm was found to be almost identical to that of several isofunctional pyrocatechases from different microorganisms[18,21], and this red coloration was assumed to be due to the mode of chelation between non-heme irons and amino acid residues

of the enzyme protein. From a number of recent studies on several isofunctional pyrocatechases[9,11,22,23], it was found that pyrocatechase having a molecular weight about 6×10^4 is a dimer composed of two non-identical subunits with 2 g atoms of non-heme iron per mole of the enzyme as a sole prosthetic group, and that the trivalent ferric iron may be responsible for the absorption of the enzyme in the visible range.[21]

The enzyme preparation of soil bacterium showed a strict substrate specificity towards catechol as in the case of pyrocatechase from *Pseudomonas arvilla* C-1[18]. Narrow substrate specificity for isofunctional pyrocatechases can be expected since the bacterial enzymes concerned with the degradation of aromatic compounds seem to be evolved independently[13,24]. However, the substrate specificity of pyrocatechases from different microorganisms shows considerable variation. Pyrocatechase from *Pseudomonas* sp. of Pseudomonadeceae showed a strict substrate specificity that no compound other than catechol and 4-methyl catechol served effectively as substrate while the enzyme from *Brevibacterium* sp. of Corynebacteria group acted on diverse analogues of catechol[10,18,24~26]. Our enzyme preparation from a soil bacterium was found to have the highest affinity towards catechol with the K_m value of $1.9\times10^{-6}M$. Although the enzyme showed an absolute substrate specificity with catechol, it was also found that the compounds having the *ortho-* or *para-*dihydroxy groups on the aromatic ring, *p*-hydroquinone and pyrogallol, exhibit marked inhibitions on the enzyme activity. By the introduction of the carboxyl group at 4-position of catechol, however, the binding affinity to the enzyme was reduced remarkably while with 4-nitro catechol the affinity of such dihydroxy group to the enzyme was rather enforced. This result suggests that the *orth-* or *para*-dihydroxy group on the aromatic ring may participate in the enzyme-substrate binding and that the binding affinity of catechol to the enzyme may also be enforced by the presence of an uncharged electron withdrawing group like a nitro group at 4-position.

The involvement of the trivalent ferric iron for the enzyme activity was also demonstrated by the inhibition study with several chelating agents. In the presence of Tiron, the enzyme activity was reduced seriously by forming a colored chelate complex while *o*-phenathroline and a, a′-dipyridyl, typical chelating agents for ferrous iron, neither inhibited the enzyme activity significantly nor formed chelate complexes with the enzyme. This result seems to indicate that the trivalent state of non-heme iron may be responsible for the enzyme activity[10,18]. By contrast, the other types of microbial dioxygenases including metapyrocatechase and 3,4-dihydroxy phenylacetate 2,3-oxygenase were reported to contain divalent iron based on the ESR spectra and the results of inhibition studies with chelating agents[13].

The effect of alkylating agents on the enzyme activity were found to be different from those reported previously with an isofunctional pyrocatechase of *Pseudomonas arvilla*[10,18]. N-Ethyl maleimide (NEM) blocked the enzyme reaction up to 90 percent while iodoacetamide (IAA) did not show any significant inhibition. It seems that NEM may access the functional group of the enzyme more readily than IAA because of its ring structure. The activation of the enzyme activity was observed by the presence of glutathion as a sulfhydryl agent. The stimulative effect by the sulfhydryl agents seems to be due to either the binding with heavy metals, the reduction of orthobenzoquinone as a possible intermediate during the autooxidation of cate-

chol, or the protection of free thiol groups acting functional groups of the enzyme. From a set of the enzyme inhibition study with sulfhydryl blocking agents, it seems to indicate that there may be more than a pair of thiol groups in the active center of the enzyme[13]. Some sulfhydryl groups may not be in free state or are in hidden state, for example as those in iron-sulfur bonds, since the addition of glutathion did not restore the enzyme activity fully.

Futher studies on the mode of inhibition and the reaction mechanism of the enzyme action will be reported in subsequent paper.

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