Characteristics of a New Catechol 1,2-Oxygenase from *Trichosporon cutaneum* WY 2-2

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Received March 24, 1981

Successive feeding of phenol at concentrations of less than 5.5 mM into a thick suspension of *Trichosporon cutaneum* WY 2-2 precultured in MPY-medium resulted in a high yield (approximately 28.7 g wet cells/liter) of intact cells capable of decomposing phenol actively (3.7 $\mu\text{mol/min/g}$ of wet cells).

The effects of pH and additions of ethanol and 2-mercaptoethanol were tested on the stability of crude extracts from the strain grown on phenol. The crude extracts were stable at a pH range of 7.6 and 8.3, and were stable for 35 days when 10% ethanol and 5 mM 2-mercaptoethanol were added.

A highly purified preparation of catechol 1,2-oxygenase was obtained from strain WY 2-2 grown on phenol. The purified enzyme was homogeneous on polyacrylamide disc-gel electrophoresis. The enzyme had a molecular weight of about 105,000 and gave rise to subunits of molecular weight of 35,000 by SDS gel electrophoresis. Therefore, the enzyme appears to be a trimer of subunits with identical molecular weight. The Michaelis constants were 9.0 μM for catechol and 6.8 μM for 4-methylcatechol. The enzyme exhibited higher activities towards 4-methylcatechol and hydroxyquinol than towards catechol, and had essentially the same substrate specificity as the crude extracts. 4-Methylcatechol completely inhibited the enzyme activity towards catechol.

Catechol 1,2-oxygenase in bacteria was originally purified from a *Pseudomonas*, and it was proved to be a dioxygenase.^{1~4)} The enzyme was isolated from *Brevibacterium fuscum*,⁵⁾ also.

On the other hand, catechol 1,2-oxygenase in fungi was highly purified from a strain of *Trichosporon cutaneum*.⁶⁾

In a previous paper,⁷⁾ we reported that catechol 1,2-oxygenase from *Trichosporon cutaneum* WY 2-2, which isolated by us from an activated sludge, was apparently different in its substrate specificity from the enzyme from *T. cutaneum* isolated by Neujahr *et al.*⁶⁾ and the other such enzyme in bacteria.^{1~5)}

This report describes an effective technique to get high yield of intact cells of *T. cutaneum* WY 2-2 capable of decomposing phenol actively, on stability of crude extracts from the strain grown on phenol and the characteristics of the enzyme purified from extracts of the strain grown on phenol.

MATERIALS AND METHODS

Organism. *Trichosporon cutaneum* WY 2-2.

Method of cultivation. *T. cutaneum* WY 2-2 was subcultured for 20 hr at 30°C with shaking in a total volume of 2 liter in 2-liter conical flasks each containing 500 ml of MPY-medium consisting of: malt extract broth (Difco Laboratories, Detroit, USA) 2.0%, Polypepton (Daigo Eiyokagaku Co., Osaka, Japan) 0.5%, yeast extract (Oriental Yeast Co., Tokyo, Japan) 0.01%. Then cells (70 gram of wet weight) were centrifuged at 8000 $\times g$ for 20 min, washed once with distilled water and adapted in a 5-liter mini-jar fermentor (L. E. Marubishi Co., Tokyo, Japan) containing 2.5 liter of the following medium (pH 6.0) consisting of: phenol 5.2 mM, NH_4Cl 0.5%, KH_2PO_4 0.25%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1%, NaCl 0.01%, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.001%, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.001%, yeast extract (Oriental Yeast Co.) 0.01% and 1 ml/liter salts solution containing (mg/liter) H_3BO_3 500, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 40, KI 100, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 200, MnSO_4 400, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ 200 and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 400. Phenol at concentrations of less than 5.5 mM was fed successively. Cultivation conditions were as follows: rotation speed, 550 rpm; aeration rate, 1 to 1 vol/vol/min (volume of air supplied/volume of culture/min); temperature, 30°C.

Determinations. Growth of *T. cutaneum* WY 2-2 was measured by a Fuji electrophotometer with Filter No. 660 and is expressed as ADS unit which was calculated from the multiplication of optical density by 130. Phenol was determined by the method of Folin *et al.*⁸⁾ Gas chromatography was carried out with a Hitachi gas chromatograph, Model K-53, equipped with a flame ionization detector operated in single-column mode under the following conditions: column, 1 m $\times$ 3 mm. I.D. stainless steel packed with 10% Silicone SE-30 on 60~80 mesh Chromosorb WAW; temperature, programmed from 100°C at 10°C/min to 240°C; flow rate of nitrogen as carrier gas, approx. 15 ml/min. Gas chromatography-mass spectrometry was carried out with a JEOL JMS-100 mass spectrometer-JGC-20K gas chromatograph equipped with an electron impact ionization system.

Enzyme assay. Catechol oxygenase activity was determined spectrophotometrically by the increase in absorbance at 260 nm except pyrogallol at 300 nm and hydroxyquinol at 245 nm with a Hitachi spectrophotometer Model 124.³⁾ One enzyme unit is defined as the amount of enzyme catalyzing the formation of 0.1 μ mol of the respective product expected (catechol to *cis,cis*-muconic acid (ϵ_{260} = 16,800), 4-methylcatechol to 3-methylmuconic acid (ϵ_{260} = 13,900),²⁰⁾ 3-methylcatechol to 2-methylmuconic acid (ϵ_{260} = 18,000),²⁰⁾ pyrogallol to 2-hydroxymuconic acid (ϵ_{296} = 14,500)²⁰⁾ and hydroxyquinol to maleylacetic acid (ϵ_{245} = 5,400)²¹⁾/min. Specific activity is calculated as enzyme units/mg of protein.

Each cuvette contained in a final volume of 1 ml: Tris-HCl buffer, pH 8.3 at 4°C, 25 μ mol; ethanol, 50 mg; 2-mercaptoethanol, 2.5 μ mol; substrate, 0.1 μ mol; enzyme solution, 0.01~0.1 ml corresponding to 0.01~2.0 mg of protein. Assay was done at room temperature.

Protein estimation. Protein concentration was determined by the method of Lowry *et al.*⁹⁾

Disc gel electrophoresis. Polyacrylamide gel electrophoresis was carried out using the "pH 8.9-7.5%" gel system described by Maurer.¹⁰⁾ After electrophoresis the gels were stained with a solution of Coomassie brilliant blue R-250 (0.25% in 7% acetic acid) to locate the protein bands.

Molecular weight estimation. The molecular weight was determined by the method of Whitaker.¹¹⁾ A column of Sephadex G-200 (2 $\times$ 80 cm) was used. Blue dextran, aldolase, serum albumin, ovalbumin and cytochrome *c* were used to calibrate the column. 50 mM Tris-HCl buffer, pH 8.0 at 4°C containing 10% ethanol, 5 mM 2-mercaptoethanol and 4 μ M FeSO₄ was employed for equilibration and elution. Polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS) and 2-mercaptoethanol was performed according to the procedure of Weber and Osborn.¹²⁾ The LMW Kit E (Pharmacia Fine Chemicals,

Uppsala, Sweden) was used as protein reference.

Chemicals. Most of the chemicals were commercially available products of reagent grade. All catechols except 4-nitrocatechol were recrystallized before use. The inorganic salts, *o*-phenanthroline, α,α' -dipyridyl, ethylenediaminetetraacetic acid (EDTA), catechol, 4-methylcatechol, 3-methylcatechol, phenol, Na-ascorbate, Tiron (catechol 3,4-disulphonate-Na), protamine sulphate, protocatechuate, 4-nitrocatechol and Tris Buffer were products of Yoneyama Yakuhin Co., Kyoto, Japan. Peroxidase (horse radish) and catalase (bovine liver) were products of Boehringer GmbH, Germany. Blue dextran, Sephadex G-200 and DEAE-Sephacel were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Bovine serum albumin (crystalline), ovalbumin (crystalline), aldolase (rabbit muscle) and cytochrome *c* (horse heart) were products of Sigma Chemical Co., St. Louis, USA. *p*CMB (*p*-chloromercuribenzoate) and 1,2-naphthoquinone were obtained from Merck AG (Darmstadt, Germany) and *p*-benzoquinone was obtained from Tokyo Kaseikogyo Co., Tokyo, Japan. *cis,cis*-Muconic acid and 3-methylmuconic acid were synthesized from catechol and 4-methylcatechol respectively, according to the method of Elvidge *et al.*¹³⁾ Diazomethane was prepared according to the method of Arndt.¹⁴⁾ Hydroxyquinol was synthesized by the method of Thiele.¹⁵⁾

RESULTS

Successive feeding of phenol to the thick suspension of Trichosporon cutaneum WY 2-2

It was tested whether successive feeding of phenol into a thick suspension of *T. cutaneum* WY 2-2 precultured in MPY-medium would result in a high yield of intact cells capable of decomposing phenol actively. Figure 1 illustrates time course of successive feeding of phenol to the thick suspension of the strain.

Seventy gram of wet cells of WY 2-2 grown in 2 liter of MPY-medium were adapted in a 5-liter mini-jar fermentor in 2.5 liter of the medium containing phenol at a concentration of 5.2 mM. After lag time of 2.5 hr, almost all of phenol was consumed and then successive feeding of phenol at concentrations of less than 5.5 mM resulted in a high yield (about 86 gram of wet cells/3 liter of the medium) of the organism. Even at the 7th feeding (after 7 hr) the culture decomposed phenol at a rate of 3.7 μ mol (347 μ g)/min/gram of wet cells and total phenol consumed was about 8.4 gram/3 liter of the medium.

FIG. 1. Time Course of Successive Feeding of Phenol to the Thick Suspension of *Trichosporon cutaneum* WY 2-2. The cells (70 gram of wet weight) precultured in 2 liter of MPY-medium and centrifuged at $8000 \times g$ for 20 min were washed once with distilled water and adapted in a 5-liter mini-jar fermentor containing 2.5 liter of medium. The concentration of phenol fed once was less than 5.5 mM. Other details are described in MATERIALS AND METHODS. Symbols: $\triangle$ —growth, ADS_{660} unit; $\circ$ —residual phenol, mM; $\diamond$ —total phenol consumed, mg/ml.

Effect of pH and additions of ethanol and 2-mercaptoethanol on stability of crude extracts from *Trichosporon cutaneum* WY 2-2

The crude extracts from *T. cutaneum* WY 2-2 were very unstable and catechol oxygenase activity in the crude extracts was inactivated at pH 7.0 in the midst of the purification process. So the effects of pH and additions of ethanol and 2-mercaptoethanol on stability of crude extracts from the strain grown on phenol were tested.

Catechol oxygenase in a crude extract was rapidly inactivated at pH 6.0 and there was a 80% inactivation after 3 days of storage at 0°C . At pH 7.0, 80% of the activity was lost after 13 days of storage at 0°C . At pH 7.6, 8.2 and 8.3, the original activity was preserved completely even after 16 days of storage at 0°C . Therefore, it appeared that the crude extract was stable in the pH range between 7.6 and 8.3.

Figure 2 indicates effects of several ad-

FIG. 2. Effects of Several Additions on Stability of Catechol Oxygenase in a Crude Extract of *T. cutaneum* WY 2-2.

The extracts were stored at 4°C .

Symbols: $\circ$ —Tris-HCl, pH 8.0 at 4°C ; $\triangle$ —Tris-HCl, pH 8.0 at 4°C + 5 mM 2-mercaptoethanol; $\diamond$ —Tris-HCl, pH 8.0 at 4°C + 10% ethanol; $\square$ —Tris-HCl, pH 8.0 at 4°C + 5 mM 2-mercaptoethanol + 10% ethanol; ∇ —Tris-HCl, pH 8.0 at 4°C + 5 mM 2-mercaptoethanol + 10% ethanol + $10 \mu\text{M}$ FeSO_4 .

ditions on stability of crude extracts at pH 8.0. The extract was stable for 20 days by the addition of 2-mercaptoethanol or ethanol alone, but was completely inactivated after 35 days of storage at 0°C . By the additions of both 2-mercaptoethanol and ethanol, the activity was preserved even after 35 days of storage at 0°C . The effects on addition of di- or trivalent iron towards the extract were uncertain.

Purification of catechol 1,2-oxygenase

All operations were carried out at 4°C , unless otherwise stated.

Step 1. Crude extract. One hundred and fifty gram of frozen (-20°C) cell paste was washed twice with 400 ml of 50 mM Tris-HCl buffer (pH 8.0 at 4°C). The wet cell paste was then suspended in 400 ml of 50 mM Tris-HCl buffer (pH 8.0 at 4°C) containing 10% ethanol, 5 mM 2-mercaptoethanol and $4 \mu\text{M}$ FeSO_4 (Buffer A). 100 ml portions of this suspension were disrupted in a Vibrogen cell mill (Edmund Bühler, Germany) with an equal volume of glass beads (0.4–0.5 mm) for 4 min,

filtrated with glass filter (17G2) in order to remove glass beads, centrifuged for 30 min at $8000 \times g$ to remove unbroken cells and then centrifuged for 90 min at $65,000 \times g$. The supernatant (557 ml) contained 756.5 mg of protein and the specific activity of the enzyme was 0.85 U/mg.

Step 2. Protamine sulfate treatment. Thirty ml of a 2% solution of protamine sulfate (pH 7.2) was added to 540 ml of the crude extracts obtained in Step 1. The mixture was agitated for 20 min. The precipitate was removed by centrifugation at $8000 \times g$ for 20 min. The supernatant obtained (562 ml) had 516.7 mg of protein and the specific activity was 1.33 U/mg of protein.

Step 3. Fractionation with $(\text{NH}_4)_2\text{SO}_4$. The supernatant from Step 2 (550 ml) was mixed with 470 ml of a saturated ammonium sulfate (pH 7.2 adjusted with NH_4OH). The precipitate formed was removed after 1 hr by centrifugation for 20 min at $8000 \times g$ and discarded. The supernatant was mixed with 1150 ml of the saturated ammonium sulfate solution and the mixture was stored overnight at 4°C . The precipitate formed was gathered by centrifugation for 90 min at $65,000 \times g$. The 45~75% ammonium sulfate precipitate was dissolved in 50 ml of Buffer A and dialyzed against the same buffer for 2 days at 4°C . The resulting extract solution (58.3 ml) contained 151.6 mg of protein and the specific activity was 7.46 U/mg of protein.

Step 4. Chromatography on a DEAE-Sephacel column. Fifty ml of the extract from Step 3 were applied to a DEAE-Sephacel column (2.5×60 cm) equilibrated with Buffer A. The elution was carried out with 350 ml of the same buffer and subsequently with an ammonium sulfate gradient (0 $\rightarrow$ 0.2 M, 1.8 liter for total volume). Absorbancy at 280 nm and enzyme activity were determined in 5 ml fractions. The most active fractions were combined (25 ml) and the enzyme was precipitated by the addition of 7 g crystalline $(\text{NH}_4)_2\text{SO}_4$. The precipitate was collected after 1 hr by centrifugation for 20 min at $8000 \times g$. It was dissolved in 2 ml of Buffer A. The enzyme

solution was dialyzed against the same buffer overnight at 4°C . The enzyme solution contained 40 mg of protein and the specific activity was 19.6 U/mg of protein.

Step 5. Chromatography on a Sephadex G-200 column. One ml of the enzyme solution obtained in the preceding step was applied to a Sephadex G-200 column (2×80 cm) equilibrated with Buffer A. The elution was run with the same buffer. A purple band indicated the movement of the enzyme. The most active fractions were pooled (12 ml). The enzyme solution obtained had 3.3 mg of protein and the specific activity was 129.9 U/mg of protein.

Step 6. Chromatography on a hydroxyapatite column. Ten ml of the enzyme solution from Step 5 were applied to a column of hydroxyapatite (1.5×10 cm) equilibrated with Buffer A without $4 \mu\text{M}$ FeSO_4 . The elution was run with the same buffer and then carried out with 40 ml of 1 mM potassium phosphate buffer (pH 7.6) containing 10% ethanol and 5 mM 2-mercaptoethanol. Subsequently, a potassium phosphate gradient (1 $\rightarrow$ 50 mM, 80 ml for total volume) was applied. The gradient contained 10% ethanol and 5 mM 2-mercaptoethanol. The most active fractions were eluted between 10 and 16 mm. The active enzyme (2 ml) was precipitated by the addition of 5.6 g crystalline $(\text{NH}_4)_2\text{SO}_4$. The precipitate was gathered after 1 hr by centrifugation for 20 min at $8000 \times g$ and it was dissolved in 6 ml of 50 mM Tris-HCl buffer (pH 7.8 at 4°C) containing 10% ethanol, 5 mM 2-mercaptoethanol and $4 \mu\text{M}$ FeSO_4 and dialyzed against the same buffer for 2 days at 4°C . The enzyme solution was preserved at -20°C . The enzyme solution contained 0.96 mg of protein and the specific activity was 181.5 U/mg of protein.

Figure 3 illustrates a chromatography of catechol 1,2-oxygenase from *T. cutaneum* WY 2-2 on a hydroxyapatite column using potassium phosphate gradient (Step 6).

The purification procedure is summarized in Table I. The overall yield was 27% of the activity present in the crude extract and the

enzyme was purified 213.5-fold.

However, the exact value of the yield as well as the degree of purification is difficult to estimate because of the activation of the enzyme upon purification. It can be seen in Table I that the total activity of the enzyme nearly doubled after the 3rd purification step.

FIG. 3. Chromatography of Catechol Oxygenase on Hydroxyapatite Column (1.5×10 cm).

Ten ml of the enzyme solution was applied to a hydroxyapatite column. First, the elution was carried out with 40 ml of Tris-HCl-EtOH-mercaptoethanol buffer (pH 7.8) and subsequently with 40 ml of 1 mM K-phosphate-10% EtOH-5 mM mercaptoethanol buffer (pH 7.6). And then a phosphate gradient (1→50 mM, total 80 ml) was applied. Flow rate: 10 ml/hr.

Symbols: —●— O.D., 280 nm; —○— activity, units/ml; ----- K-phosphate, mM.

Homogeneity and molecular weight

The molecular weight of the enzyme was found to be approximately 105,000 as calculated from two different determinations. Figure 4 indicates a chromatography of catechol oxygenase on Sephadex G-200.

The enzyme treated by heating at 100°C for 5 min in a 1% Sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol solution, showed a

FIG. 4. Plot of Standard Proteins and Catechol Oxygenase (CO) Chromatographed on Sephadex G-200 (2×80 cm).

CO: catechol oxygenase; 1, cytochrome *c* (12,000); 2, ovalbumin (43,000); 3, serum albumin (67,000); 4, aldolase (158,000); 5, blue dextran (2,000,000).

TABLE I. PURIFICATION OF CATECHOL 1,2-OXYGENASE FROM *T. cutaneum* WY2-2 GROWN ON PHENOL

Step No.	Enzyme in	Volume (ml)	Protein (mg/ml)	Specific activity (units/mg)	Total protein (mg)	Total activity (units)	Purification (fold)	Yield (%)
1	Crude extract	557.0	1.4	0.85	756.5	643.0	1.0	100
2	Protamine treated supernatant	562.0	1.0	1.33	576.7	767.0	1.6	119
3	Ammonium sulphate precipitate 45 to 75%	58.3	2.6	7.46	151.6	1130.9	8.8	176
4	DEAE Sephacel treated enzyme	25.0	1.6	19.6	40.0	784.0	23.1	122
5	Sephadex G-200 passed enzyme	10.0	0.33	129.9	3.3	428.6	152.8	67
6	Eluate from a hydroxy-apatite column	8.0	0.12	181.5	0.96	174.2	213.5	27

FIG. 5. Estimation of Molecular Weight of Catecho Oxygenase by SDS-Gel Electrophoresis

Sample treatment: All proteins (A and B) except C—1% SDS, 5% 2-mercaptoethanol, 100°C for 5 min.

A: reference proteins; B: catechol oxygenase, heated; C: catechol oxygenase, not heated. CO: molecular weight of catechol oxygenase, 35,000.

Molecular weight of reference proteins: 1. rabbit muscle phosphorylase b, 94,000; 2. bovine serum albumin, 67,000; 3. ovalbumin 43,000; 4. bovine erythrocyte carbonic anhydrase, 30,000; 5. soybean trypsin inhibitor, 20,100; 6. α -lactalbumin, 14,400.

single band on SDS-polyacrylamide gel electrophoresis and the molecular weight of the protein band was calculated to be approximately 35,000 as shown in Fig. 5. Therefore, this result suggests that the enzyme protein consists of three subunits of the same molecular weight.

Substrate saturation

The Michaelis constant, K_m , was found to be $9.0 \mu\text{M}$ for catechol and $6.8 \mu\text{M}$ for 4-methylcatechol, as determined from Lineweaver-Burk plots¹⁶⁾ of reciprocal reaction velocities versus reciprocal substrate concentrations.

The effect of heavy metals and chelating agents

Among the heavy metal derivatives, HgCl_2 (87% inhibition) and $p\text{CMB}$ (75% inhibition) were the most potent inhibitors. The potent chelating agents, in decreasing order of inhibition, were *o*-phenanthroline (64.7% in-

TABLE II. THE EFFECT OF HEAVY METALS AND CHELATING AGENTS ON THE ACTIVITY OF PURIFIED CATECHOL 1,2-OXYGENASE FROM *T. cutaneum* WY 2-2

Addition	Concn. (mM)	Enzyme activity (%)
None	1.0	100
AgNO_3	1.0	72.6
HgCl_2	1.0	13.0
$p\text{CMB}$	1.0	24.7
CuSO_4	1.0	53.5
Tiron	1.0	43.8
	0.1	97.1
α, α' -Dipyridyl	1.0	5.7
	0.1	87.8
<i>o</i> -Phenanthroline	0.1	35.3
	0.01	68.5
EDTA	1.0	100.0
Na_2AsO_3	1.0	84.1
NaN_3	1.0	95.5
KCN	1.0	46.6

Spectrophotometric assay: $p\text{CMB}$, *p*-chloromercuribenzoate; Tiron, 4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt; EDTA, ethylenediaminetetraacetic acid.

hibition, 0.1 mM), α, α' -dipyridyl (94.0% inhibition, 1.0 mM) and Tiron (56.0% inhibition, 1.0 mM). KCN (1 mM) showed 53% of inhibition. The effect of heavy metals confirms the involvement of SH-groups, and that of the iron chelating agents indicates the presence in the enzyme of both, di- and trivalent iron (Table II).

The effect of quinones and oxidizing and reducing agents

Oxidizing agents were without effect, but reducing agents strongly inhibited the enzyme activity. Ascorbate and the quinones examined were powerful inhibitors (Table III).

The effect of substrate analogues

The effects of substrate analogues as inhibitors of the enzyme activity towards catechol were determined (Table IV). The enzyme activity was inhibited completely by 4-methylcatechol and also inhibited to considerable extents by other catechols except protocathechuate. Inhibition by these catechols may be

TABLE III. THE EFFECT OF QUINONES AND OXIDIZING AND REDUCING AGENTS ON THE ACTIVITY OF PURIFIED CATECHOL OXYGENASE FROM *T. cutaneum* WY 2-2

Addition	Concn. (mM)	Enzyme activity (%)
None		100.0
<i>p</i> -Benzoquinone	1.0	0
1,2-Naphthoquinone	1.0	38.6
Dithionite	1.0	77.1
Ascorbate	1.0	0
Peroxidase (1.75 mg)		90.0
Catalase (32,000 U/0.5 mg)		98.6

Spectrophotometric assay. Substrate: 0.1 mM catechol. The sequence of additions of the reaction components was: enzyme+the compound to be tested+substrate.

TABLE IV. ACTIVITY OF CATECHOL 1,2-OXYGENASE WITH VARIOUS CATECHOL DERIVATIVES AND INHIBITION OF THE OXIDATION OF CATECHOL BY THESE COMPOUNDS

Activity was spectrophotometrically determined using 0.1 μ mol of each compound as described in the text. To measure the rate of catechol oxidation in the presence of an inhibitor, the latter was added to reaction mixture immediately before addition of catechol. Inhibition (%) is shown as percentages of the oxygenase activity towards catechol alone.

Compound	Activity (%)	Inhibition (%)
Catechol	100.0	
4-Methylcatechol	185.8	100.0
3-Methylcatechol	6.2	44.4
Hydroxyquinol	159.0	51.6
Pyrogallol	8.5	49.2
4-Nitrocatechol	0	54.0
Protocatechuate	0	0

due to competition as substrate for catalytic sites.

Substrate specificity

As shown in Table IV, the highly purified enzyme exhibited higher activities towards 4-methylcatechol and hydroxyquinol than towards catechol, but it also catalyzed the oxidation of 3-methylcatechol and pyrogallol a

little. And it had essentially the same substrate specificity as the crude extracts.

Identification of the oxidation products of catechol and 4-methylcatechol

As mentioned before, *T. cutaneum* WY 2-2 catechol oxygenase differs in substrate specificity from other known catechol oxygenases. Therefore, it was necessary to check whether the mode of action of this enzyme was similar to that of other catechol oxygenases. Experiments were conducted to isolate and identify the oxidation products of catechol and 4-methylcatechol.

Successive portions of catechol or 4-methylcatechol (100 mg in total) were added to a solution of 100 ml of Buffer A and 20 mg of protein of the partially purified enzyme preparation taken after Step 4 of purification (Table I) in such a way that the concentration of catechol or 4-methylcatechol was maintained at approximately 0.5 mM. The reaction was carried out at room temperature with constant stirring. Absorbance of aliquots were read at 260 nm at 30 min intervals. After 5 hr, the reaction mixture was freeze-dried at -80°C overnight and then it was freeze dried.

The oxidation products were isolated by the thin layer chromatography from the ethanol extracts of freeze-dried samples and methylated by diazomethane. The methyl esters of the oxidation products were gas-chromatographed with programmed-temperature and they were analyzed on a JEOL JMS-100 double focusing spectrometer with a JEOL JGC-20K gas chromatograph.

The results of programmed-temperature gas chromatography of methyl esters of the oxidation products were in agreement with dimethyl esters of authentic *cis,cis*-muconic acid and 3-methylmuconic acid, respectively.

Mass spectra of methyl esters of the oxidation products of catechol and 4-methylcatechol are shown in Figs. 6 and 7. The spectra showed molecular ion peaks at m/z 170 corresponding to $\text{C}_8\text{H}_{10}\text{O}_4$, dimethyl ester of *cis,cis*-muconic acid, and m/z 184 corresponding to $\text{C}_9\text{H}_{12}\text{O}_4$, dimethyl ester of 3-methyl-

FIG. 6. Mass Spectra of Dimethyl Ester of Authentic *cis,cis*-Muconic Acid (A) and Methyl Ester of the Oxidation Product of Catechol (B).

FIG. 7. Mass Spectra of Dimethyl Ester of Authentic 3-Methylmuconic Acid (A) and Methyl Ester of the Oxidation Product of 4-methylcatechol (B).

muconic acid, respectively, and also prominent peaks of fragmentation were in agreement with that of authentic samples (Figs. 6 and 7).

Previously, Nakagawa *et al.* confirmed that the purified catechol 1,2-oxygenase from *Brevibacterium fuscum* was converted catechol, 4-methylcatechol, 3-methylcatechol and pyrogallol to *cis,cis*-muconic acid with $\lambda_{\text{max}}^{\text{pH } 7.0}$

at 258 nm, 3-methylmuconic acid with $\lambda_{\text{max}}^{\text{pH } 7.0}$ at 265 nm ($\lambda_{\text{max}}^{\text{N KOH}}$ at 265 nm), 2-methylmuconic acid with $\lambda_{\text{max}}^{\text{pH } 7.0}$ at 266 nm ($\lambda_{\text{max}}^{\text{N KOH}}$ at 266 nm) and 2-hydroxymuconic acid with $\lambda_{\text{max}}^{\text{pH } 7.0}$ at 296 nm, respectively.⁵⁾

Moreover, Chapman and Ribbons described that maleylacetic acid with $\lambda_{\text{max}}^{\text{pH } 7.0}$ at 245 nm in 50 mM phosphate buffer was identified as the product of hydroxyquinol oxidation using the crude extracts derived from resorcinol-grown cells of *Pseudomonas putida*.²¹⁾

The reaction products of catechol, 4-methylcatechol, 3-methylcatechol, pyrogallol and hydroxyquinol with our purified enzyme in 50 mM Tris-HCl buffer (pH 7.5) showed probably the spectra of *cis,cis*-muconic acid with $\lambda_{\text{max}}^{\text{pH } 7.5}$ at 258 nm, 3-methylmuconic acid with $\lambda_{\text{max}}^{\text{pH } 7.5}$ at 263 nm, 2-methylmuconic acid with $\lambda_{\text{max}}^{\text{pH } 7.5}$ at 268 nm, 2-hydroxymuconic acid with $\lambda_{\text{max}}^{\text{pH } 7.5}$ at 296 nm and maleylacetic acid with $\lambda_{\text{max}}^{\text{pH } 7.5}$ at 245 nm, which were omitted in this paper, respectively. And they also gave the same ultraviolet absorption as crude extracts from *T. cutaneum* WY 2-2.⁷⁾

Consequently, the above results show that the enzyme from *T. cutaneum* WY 2-2 is a typical "intradiol dioxygenase" giving not only *cis,cis*-muconic acid from catechol and 3-methylmuconic acid from 4-methylcatechol, but probably maleylacetic acid from hydroxyquinol, 2-methylmuconic acid from 3-methylcatechol and 2-hydroxymuconic acid from pyrogallol.

DISCUSSION

Successive feeding of phenol at concentrations of less than 5.5 mM to the thick suspensions of our strain WY 2-2 resulted in a high yield (28.7 gram of wet cells/liter) of intact cells capable of decomposing phenol actively. On the other hand, Neujahr *et al.* reported that the yield of cultivation in a tank was 3.6 gram of wet cells/liter.⁶⁾ Therefore, the author considers that successive feeding of the phenolic substrate to the thick suspensions of the microorganism precultured in MPY-

medium is a more effective technique to get high yield of intact cells capable of decomposing the substrate actively in short time.

The crude extracts from strain WY 2-2 were stable in the presence of both ethanol (10% vol/vol) and 2-mercaptoethanol (5 mM) into the buffer and the activity remained unchanged for 35 days at 0~4°C. It is assumed that addition of ethanol in the buffer protects the hydrophobic bonds in the protein by preparing for the non-polar circumstances, and that ethanol interacts 2-mercaptoethanol in the extracts by repressing the oxidation of the latter. Consequently, 10% (vol/vol) ethanol and 5 mM 2-mercaptoethanol were included in all buffers used in the purification.

The catechol 2,3-oxygenase, an extradiol type of ring fission enzyme, isolated by Kojima *et al.*¹⁷⁾ became known as the enzyme stabilized by organic solvents such as ethanol or acetone, and recently Patel and Barnsley reported that 1,2-dihydroxynaphthalene oxygenase isolated from *Ps. putida* was stabilized by the addition of both ethanol and 2-mercaptoethanol.¹⁸⁾

Investigations on the characteristics of catechol 1,2-oxygenases purified hitherto revealed that the molecular weight of the enzymes is in the range of 70,000~110,000, and that the active enzymes have distinct red colour and are non-heme iron proteins.

Similarly to the other catechol 1,2-oxygenases, the molecular weight of the enzyme purified by the author was approximately 105,000 and the characteristic red colour of the active enzyme disappeared upon inactivation.

Some significant differences between our enzyme and those isolated from other microorganisms seem to exist. Namely, the enzyme had a subunit of molecular weight of 35,000, and this result suggests that the native enzyme (105,000), in contrast to the other catechol 1,2-oxygenases,^{1,5,6)} is a trimer. The purified enzyme exhibited higher activities towards 4-methylcatechol (186%) and hydroxyquinol (159%) than towards catechol (100%), 3-methylcatechol (6%) and pyrogallol (9%), and

it was obviously different in the substrate specificity from those isolated from *Brevibacterium*⁵⁾ and *Trichosporon cutaneum*.⁶⁾

All of the catechol 1,2-oxygenases so far purified and characterized contained non-heme iron as a sole cofactor.¹⁹⁾ The enzyme activity was inhibited 53% with KCN (1 mM), which might suggest that the enzyme, differently from other ring-cleaving oxygenases, is a heme iron protein.

Acknowledgment. The author thanks Dr. F. Uchino for his valuable advice.

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