J. Microbiol. Biotechnol. (2000), **10**(1), 35–42

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Reaction Characteristics of 4-Methylcatechol 2,3-Dioxygenase from *Pseudomonas putida* SU10

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Received: August 28, 1999

Abstract Reaction characteristics of 4-methylcatechol 2,3-dioxygenase (4MC23O) purified from *Pseudomonas putida* SU10 with a higher activity toward 4-methylcatechol than catechol or 3-methylcatechol were studied by altering their physical and chemical properties. The enzyme exhibited a maximum activity at pH 7.5 and approximately 40% at pH 6.0 for 4-methylcatechol hydrolysis. The optimum temperature for the enzyme was around 35°C, since the enzyme was unstable at higher temperature. Acetone (10%) stabilized the 4MC230. The effects of solvent and other chemicals (inactivator or reactivator) for the reactivation of the 4MC230 were also investigated. Silver nitrate and hydrogen peroxide severely deactivated the enzyme and the deactivation by hydrogen peroxide was mainly due to the oxidation of ferrous ion to ferric ion. Some solvents acted as an activator and protector for the enzyme from deactivation by hydrogen peroxide. Ascorbate, cysteine, or ferrous ion reactivated the deactivated enzyme by hydrogen peroxide. The addition of ferrous ion together with a reducing agent fully recovered the enzyme activity and increased its activity about 2 times.

Key words: 4-Methylcatechol 2,3-dioxygenase, catechol, catechol 2,3-dioxygenase, inactivation, reactivation

Accumulation of pollutants, especially aromatic compounds, creates severe environmental problems, mainly because most of these aromatic compounds are thermally and chemically nondegradable thus making them persistent. One of the degrade attractive approaches to removing them from the environment is to degrade them enzymatically or microbially [24, 25]. In the enzymatic degradation of aromatic compounds such as monocyclic and polycyclic compounds, they are converted to catechol intermediates and dihydroxyaromatics with two hydroxyl substituents to adjacent aromatic carbons in one of the aromatic rings, respectively [6]. Aromatic ring fissions of catechol intermediates and dihydroxyaromatics are catalyzed by dioxygenases which incorporate both atoms of dioxygen into the substrates [21, 26].

Dioxygenases, including the catechol dioxygenases, are the enzymes that cleave the double bonds of aromatic compounds adjacent to hydroxyl groups. They are involved in a series of biodegradation reactions of aromatic pollutants, including the cleavage of aromatic rings. The catechol dioxygenases, which catalyze the critical step in the biodegradation pathway of aromatic compounds by ring fission of catechol, are classified as intradiol and extradiol catechol dioxygenases. Intradiol catechol dioxygenase cleaves the aromatic ring between two hydroxylated carbons in an intradiol fashion, whereas extradiol catechol dioxygenase cleaves between the hydroxylated carbon and adjacent nonhydroxylated carbon in the aromatic ring [22, 23, 28]. It has been known that these enzymes cleave the catechol ring with a very strict specificity either in the intradiol or in the extradiol manner [20, 21].

Catechol 2,3-dioxygenase (C23O) is one of extradiol catechol dioxygenases that contains Fe (II) as a cofactor, and cleaves catechol to the semialdehyde in an extradiol fashion [21]. The bacteria such as *Pseudomonas* sp. which produce catechol dioxygenases are grown successfully on benzoate as a sole carbon source and an inducer [20]. Catechol 2,3-dioxygenase also has the substrate specificity for catechol and its derivatives; 4-methylcatechol, 3-methylcatechol, and dihydroxycatechol. Wallis and Chapman [28] isolated a catechol 2,3-dioxygenase which preferentially acted on 3-methylcatechol rather than catechol. In our previous paper [5], catechol 2,3-dioxygenase, which reacts more actively with 4-methylcatechol than catechol or 3-methylcatechol, was purified from *Pseudomonas putida*

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SU10. Chae and Yoo [4] used *Pseudomonas putida* cells for producing the catechol in a two-phase system. We also purified catechol 2,3-dioxygenase from toluene-grown cells of *Achromobacter xylosoxidans* KF701, catalyzing cleavage reactions on catechol, 3-methylcatechol, 4-methylcatechol, and 4-fluorocatechol [17].

To enzymatically remove the aromatic compounds from the environment, a study on the enzyme properties of C23O is an important step for application. Thus, reaction characteristics of catechol 2,3-dioxygenase purified from *P. putida* SU10, which preferentially reacts on 4-methylcatechol (called 4-methylcatechol 2,3-dioxygenase; 4MC23O), were investigated in terms of pH, temperature, solvent, and chemicals (inactivator or reactivator).

Materials and Methods

Materials

The bacterial strain, *Pseudomonas putida* SU10, used in this study was isolated from sewage with *p*-toluic acid as a major carbon source [5, 24]. The cultivation conditions of *P. putida* SU10 and purification methods of 4MC23O (4-methylcatechol 2,3-dioxygenase) were the same as in our previous paper [5], unless otherwise stated. *o*-Phenanthroline was purchased from Aldrich Co. (Milwaukee, WI, U.S.A.). All other chemicals were of the analytical grade.

Assay of 4MC23O Activity

The 4-methylcatechol 2,3-dioxygenase (4MC23O) activity was measured spectrophotometrically in 50 mM of phosphate buffer (pH 7.5) containing 0.5 mM substrate, as in our previous paper [5]. 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 cleaved product of 4-methylcatechol by 4MC23O was 2-hydroxy-5-methyl-6-oxohexa-2,4-dienoic acid (HMOH-dienoic acid) and its λ_{max} for spectrophotometric absorbance was 382 nm. The molar concentration of the product was calculated from an observed absorbance level at 382 nm with its molar extinction coefficient (33,200 $M^{-1}cm^{-1}$) [28]. Specific activity of the enzyme was defined as unit(s) per mg of proteins. Protein concentration was determined according to the method of Lowry *et al.* [14], using bovine serum albumin as a reference protein.

pH Effect on 4MC23O

After the 4MC23O was purified from frozen cells of *P. putida* SU10 with multi-steps of chromatographies [5], the optimum pH of the 4MC23O activity was investigated. The following buffers were used for different pHs; citrate buffer for pHs 4.0, 5.0, and 6.0; phosphate buffer for pHs 6.0, 7.0, and 8.0; Tris/hydrochloric acid buffer for pHs 7.5, 8.0, and 9.0; glycine buffer for pHs 9.0 and 10.0. Each buffer concentration was 200 mM, in which the enzyme was reacted with 200 μM of 4-methylcatechol for 5 min at 35°C. Enzyme concentration for the reaction was 20 μM, unless otherwise described, assuming the molecular weight of the enzyme to be 120,000 daltons [5]. For test of the enzyme stability without the substrate for 13 h at 4°C at different pHs, the enzyme was incubated at various pHs with the same buffers mentioned above, and then the temperature was elevated to 35°C. After addition of 4-methylcatechol at 20 μM into the enzyme mixture, hydrolysis was performed for 2 h. The resulting reaction product, HMOH-dienoic acid, was analyzed to evaluate the enzyme stability in different pHs.

Temperature Effect

To analyze the effect of temperature on the enzyme activity and stability of purified 4MC23O, the amounts of product at different temperatures ranging from 10°C to 70°C at pH 7.5 were determined. For determining the enzyme activity, the initial reaction velocity (v_0) of 4MC23O was determined within 5 min. The total amount of HMOH-dienoic acid produced by the enzyme for a 30-min reaction was plotted as the thermal stability of enzyme. The enzyme and substrate concentrations were the same as in the pH studies.

Effect of Organic Solvents

In order to investigate solvent effect on 4MC23O stability, enzymes were incubated in 10% solvent solution (acetone, propanol, and ethanol and methanol) in 50 mM of phosphate buffer (pH 7.5) with the substrate (200 μM) at 35°C. After a 3 h reaction, amounts of product were spectrophotometrically determined. After selecting the best organic solvent to stabilize 4MC23O, the effect of 0, 10, 20 along with 30% solvent concentration on the activity was also determined at 35°C.

Metal Ion Effect on 4MC23O

The activity or stability of some enzymes are affected by metal ions, especially the metaloenzymes, and in particular catechol dioxygenase. Here, the effects of various metal ions such as iron, copper, magnesium, manganese, strontium, cobalt, zinc, silver, nickel, mercury, and calcium ion on the 4MC23O were investigated, by determining HMOH-dienoic acid produced after incubating the enzyme-substrate (20 μM–200 μM) solution with each metal ion for 30 min at 25°C and pH 7.5. The concentration of the metal ions was 0.1 mM.

Inactivation and Protection of 4MC23O

Using silver nitrate ($AgNO_3$) and hydrogen peroxide (H_2O_2) chosen as sulfhydryl group inhibitor and oxidizing agent [18, 21], respectively, inactivation of 4MC23O was studied. After incubation of the enzyme (20 μM) with

30 μM of each deactivator for 10, 20, and 30 min, residual activities were determined by assaying the products as per standard conditions (200 μM of 4-methylcatechol, pH 7.5, in 50 mM phosphate buffer at 35°C). The effect of hydrogen peroxide concentration on the enzyme deactivation was studied with 30, 60, and 120 μM of hydrogen peroxide, and effects of the substrate and enzyme concentrations were also studied.

To investigate the protective effects of some solvents from deactivation by hydrogen peroxide, the enzyme in the same buffer containing 10% of each solvent (acetone, methanol, ethanol, and propanol) and 1 mM of *o*-phenanthroline [21] were initially treated with 30 μM of hydrogen peroxide and then incubated for 30 min at 25°C. After the incubation, the residual activities of 4MC23O were assayed under the standard conditions.

Activation and Reactivation Studies of the 4MC23O

With cysteine, ammonium ferrous sulfate [$Fe(NH_4)_2(SO_4)_2$], and ascorbic acid as reducing agents, and ferrous divalent ion as an antioxidizing agent, respectively, activation effects and their reactivation capabilities of each agent on 4MC23O activity were examined. The native enzyme solutions (20 μM) were dissolved in ascorbate (1 mM), cysteine (1 mM), ammonium ferrous sulfate (1 mM of Fe^{2+}), ascorbate and ferrous ion mixture (1 mM of each), and cysteine and ferrous mixture (1 mM of each) solutions in phosphate buffer (pH 7.5), and the reaction mixtures were incubated for 30 min at 25°C. After 200 μM substrate was added, the 4MC23O activities in these solutions for 15 min were determined under the standard conditions and compared with the control.

For the reactivation study, similar reaction procedures were carried out by the same methods, using 4MC23O deactivated by 30 μM of hydrogen peroxide. The effect of *o*-phenanthroline on the reactivation of this deactivated enzyme in the presence of 1 mM of ascorbate, cysteine, and Fe^{2+} was studied by incubating the reaction mixture with *o*-phenanthroline for 10, 20, and 30 min at 25°C.

RESULTS AND DISCUSSION

Effects of pH and Temperature on 4-Methylcatechol 2,3-Dioxygenase

The effect of pH on the activity of 4-methylcatechol 2,3-dioxygenase (4MC23O) with 4-methylcatechol was determined at 35°C in various pH buffers ranging from 4 to 10, as shown in Fig. 1. The enzyme exhibited maximum activity at pH 7.5 and the activity declined to approximately 40% at pH 6.0. The enzyme was relatively unstable at acidic pH. The optimum pH of catechol 2,3-dioxygenase (C23O) from *P. putida* mt-2 was found to be 6.5 [18]. However, extradiol non-haem iron

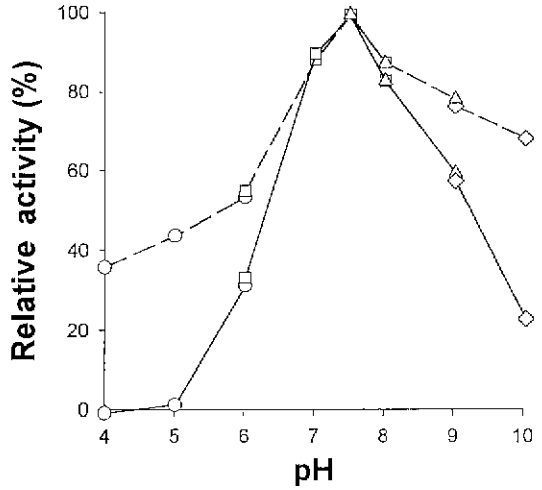

Fig. 1. Effects of pH on activity and stability of 4MC23O.
Citrate buffer for pH 4.0–6.0 (○), phosphate buffer for pH 6.0–8.0 (□), Tris/HCl buffer for pH 7.5–9.5 (△), and glycine buffer for pH 9–10 (◇) were used. Enzyme activities (—) were determined by the initial velocity (v_0) under the standard conditions (35°C, 15 min reaction 200 μM of 4-methylcatechol, and 20 μM enzyme). For the stability test (- - -), enzyme was firstly incubated in each buffer at 4°C for 13 h and then enzyme productivity was plotted by determining the amount of HMOH-dienoic acid produced for a 2-h reaction. Concentration of each buffer was 200 mM and assay was done at 35°C. The observed activities at pH 6.0 were not the same between citrate and phosphate buffers, but relative activities at this pH were represented as a single point. This is the same as at pHs 7.5, 8.0, and 9.0.

dioxygenase from *P. putida* ATCC23973 exhibited a bell-shaped pH profile, with pH 7.5 corresponding to the optimum [15].

The temperature dependence of 4MC23O activity towards 4-methylcatechol was also determined at pH 7.5

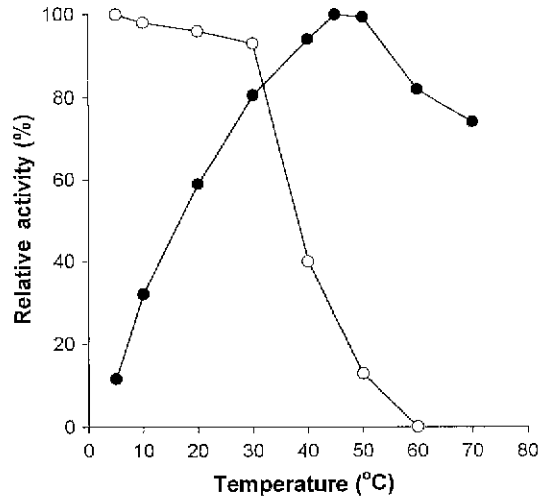

Fig. 2. Effects of temperature on the 4MC23O activity and thermal stability of the enzyme.
Temperature stabilities (○) were determined by observing the residual activities after incubating the enzyme for 30 min at each temperature in 50 mM phosphate buffer, pH 7.5. Residual activities (●) were measured under the standard condition at pH 7.5 and 35°C.

by measuring its initial velocity, v_0, at various temperatures. The activity of 4MC23O was also dependent on temperature (Fig. 2). Even if 4MC23O activity increased up to 50°C, thermostability of the enzyme for 30 min was found to be unstable at 40°C. Therefore, the maximal activity of the enzyme was observed over a range of temperature values at 35°C. Above 50°C, the enzyme easily lost its activity, indicating that the enzyme was not thermostable.

Organic Solvent Effect on 4MC23O Productivity

Generally, the enzymes are very unstable in organic solvents, because in water miscible organic solvents, hydrogen bonds between water and protein molecules are disrupted to cause unfolding of proteins. In a high concentration of organic solvent, hydrophobic interactions of protein collapse to become random coil structures [7]. However, in the present study, 4MC23O produced more HMOH-dienoic acid in a little amount of solvent (10%) than in water solution (Fig. 3A). When the enzyme was reacted with 4-methylcatechol in 10% acetone, ethanol, or propanol, the enzyme produced more product, while 10% of methanol decreased the productivity of the enzyme (Fig. 3A). It was not unusual that an enzyme was more stable

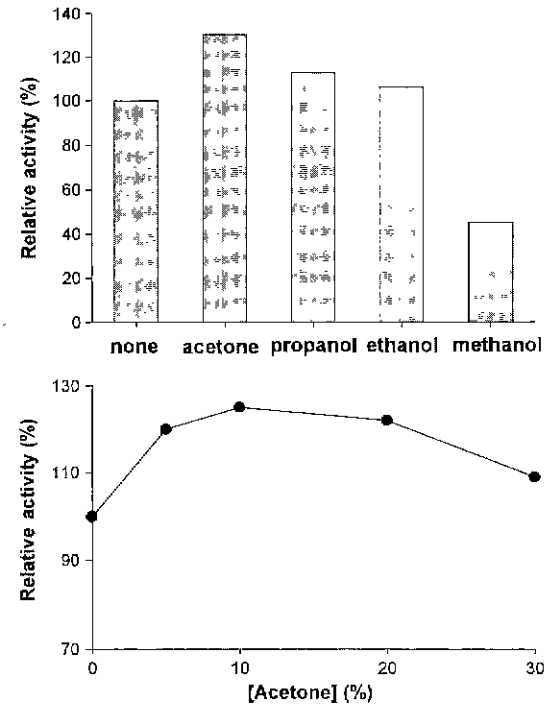

Fig. 3. Effect of organic solvents on the stabilization of 4MC23O.

(**A**) The enzymes (20 μM) were incubated with substrate (200 μm) in each solvent (10% solvent in 50 mM phosphate buffer, pH 7.5) at 25°C. Relative enzyme activities represented the amount of 2-hydroxy-5-methyl-6-oxohexa-2,4-dienoic acid (HMOH-dienoic acid) produced during the 3 h reaction against control (**B**) Effects of acetone concentration on 4MC23O stability. The conditions were the same in (**A**).

with a small amount of organic solvent. Some enzymes which catalyze nonpolar compounds, such as aromatic compounds or long chain aliphatic compounds (lipids), were reported to be stable in organic solvents [8, 10]. In particular, lipases which catalyzed hydrolysis of the ester bond of glycerides with long chain fatty acids, were very stable in highly nonpolar solvents (water immiscible organic solvent) [9, 12].

The data showed that the productivity of 4MC23O increased with an increase of nonpolarity of solvent in a 10% solution. Acetone, which is characterized to be more nonpolar than propanol, ethanol, and methanol, shows the highest productivity rate. Propanol, the second most nonpolar solvent, showed a higher activity compared to ethanol and methanol. However, it is still unclear as to why the productivity of 4MC23O increased with acetone, propanol, and ethanol. In a case of 10% methanol, which is more polar than the other solvents, productivity was lower than the control. Thus, we further investigated the effects of increasing nonpolarity of solvent on the 4MC23O productivity by changing the concentration of acetone. The data showed that productivity increased only up to 10% of acetone and decreased thereafter. When the solvent concentration was higher than a certain point, decrease of the productivity can be seen, because the enzyme should be destabilized by hydrophobic collapse [7]. Nakanishi *et al.* [19] reported that catechol 2,3-dioxygenase purified from *Pseudomonas* sp. FK-8-2 showed the highest activity when 5% acetone solution was added.

Metal Ion Effect on 4MC23O Activity

Catechol dioxygenase is a haem-containing enzyme with a metal-ion-dependent activity [21]. Many enzyme kinetic studies conducted on extradiol dioxygenase indicated evidence that C23O required ferrous ion for its enzyme activity. The bound ferrous ion (Fe^{2+}) in the active site of C23O acted as a catalyst for the cleavage of catechol ring in an extradiol fashion [26]. As shown in Table 1, the 4MC23O was affected by the metal ions. Ferrous ion ($Fe(NH_4)_2(SO_4)_2$ and $FeCl_2$), magnesium ion (Mg^{2+}), and Sr^{2+} ions all activated the enzyme activity of 4MC23O whereas zinc (Zn^{2+}), cobalt (Co^{2+}), and manganese (Mn^{2+}) ions slightly inhibited its activity. In metal ion catalysis of metaloenzymes, metal ions such as ferrous or calcium ions were known to be activators, since they induced the enzyme conformation to stabilize the bound Fe^{2+} or to assist the orientation of catalysis site for substrate binding [13, 26, 27]. However, ferric ions such as ferric sulfate and ferric chloride inhibited the 4MC23O productivity.

Silver ($AgNO_3$) and mercury ($HgCl_2$) ions deactivated the enzyme completely. Silver and mercury (mercury chloride or *p*-chloromercuribenzoate) ions are known as sulfhydryl inhibitors [21], thus these groups were thought to inactivate 4MC23O by binding to the sulfhydryl groups

Table 1. Effect of metal ions on the 4MC23O activity at 35°C for 30 min.

Metal ion	Relative activity (%)[1]	
	Ion concentration (mM)	
	0.1	1.0
Control	100	100
$Fe_2(SO_4)_3$	63	54
$FeCl_3$	59	52
$Fe(NH_4)_2(SO_4)_2$	101	112
$FeCl_2$	100	109
$CuSO_4$	72	42
$CuCl_2$	72	42
$MgSO_4$	93	106
$MgCl_2$	93	106
$MnSO_4$	87	90
$MnCl_2$	87	90
$SrCl_2$	100	106
$CoCl_2$	93	87
$ZnSO_4$	93	78
$ZnCl_2$	96	93
$AgNO_3$	0	0
$NiCl_2$	93	57
$HgCl_2$	0	0
$CaCl_2$	100	18

[1]4-Methylcatechol 2,3-dioxygenase activity without any metal ion was the basis of comparison (100%).

in enzymes. Up to the present, however, there was not even a single evidence to show that sulfhydryl groups were directly involved in the catalytic site, but rather to indicate that sulfur compounds were involved in maintaining the structure of iron-haem binding [16].

Inactivation of 4MC23O and Protection against Inactivation

As mentioned above, 4MC23O was easily deactivated by the sulfhydryl binding inhibitor and oxidized trivalent ion (Fe^{3+}). Therefore, an inactivation study was performed with an oxidizing agent (hydrogen peroxide, H_2O_2) and $AgNO_3$ (Fig. 4). Most of the enzyme activity was lost within 20 min at 25°C in the presence of H_2O_2 and $AgNO_3$ under the aerobic condition. Hydrogen peroxide was stronger for deactivation than silver nitrate. These results were consistent with data obtained with metapyrocatechase (now called as catechol dioxygenase) [21].

The hydrogen peroxide oxidized ferrous ion to ferric ion as a trivalent state. It was observed in an x-ray crystallography study that the active enzyme of C23O changed to an inactive conformation by oxidized ferric ion [26], this trivalent ferric ion deactivating the 4MC23O. In 20 μM of enzyme concentration, 30 μM of hydrogen peroxide was enough for deactivation. At 40 μM, which was a higher concentration level than that of enzyme, a larger amount of hydrogen peroxide was required to complete the deactivation process (data not shown). In

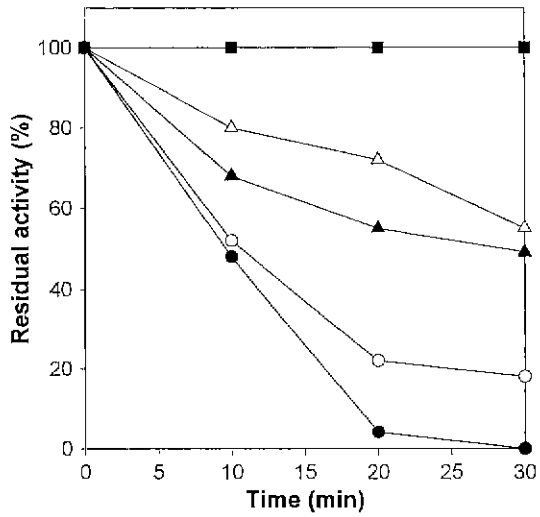

Fig. 4. Inactivation of 4MC23O by H_2O_2 and $AgNO_3$.
Inactivation of 4-methylcatechol 2,3-dioxygenase (20 μM) was carried out by incubating enzyme at 25°C with the substrate (200 μM) in phosphate buffer (50 mM, pH 7.5) and by adding 30 μM of H_2O_2 (●), 30 μM of $AgNO_3$ (○), and no addition as a control (■). Inactivation studies for 4MC23O by changing the substrate and enzyme concentrations were done: △, 30 μM of H_2O_2, 20 μM of enzyme and 1 mM of substrate; ▲, 30 μM of H_2O_2, 40 μM of enzyme, and 200 μM of substrate.

fact, more than 50% of the enzymes out of 40 μM of 4MC23O remained as the active form with 30 μM of hydrogen peroxide (Fig. 4). This suggested that at least an equivalent amount of hydrogen peroxide was required for deactivation.

Enzyme inhibition or denaturation/deactivation was mostly delayed with an increase of substrate by specifically binding the substrate [11]. Figure 4 shows that when the enzyme was incubated with substrate (1 mM of 4-methylcatechol) and hydrogen peroxide, it seemed less deactivated by hydrogen peroxide. Only 20% of the 4MC23O activity was deactivated when 1 mM of 4-methylcatechol and hydrogen peroxide were added simultaneously to the reaction mixture and incubated for 15 min, while 95% of the enzyme were inactive without the substrate. Nozaki *et al.* [21] reported that metapyrocatechase was rarely inactivated with 1 mM catechol by H_2O_2. In general, the substrate combines with enzyme in the active site rather than in a nonspecific way, and thus stabilizes enzyme either by its correct proximity and orientation or conformational change of protein to avoid inactivation [13]. Senda *et al.* [26] concluded that small conformational changes occurred in the crystal structure of dioxygenase around the active site upon substrate binding.

Protection against Inactivation of 4MC23O by H_2O_2

Inactivation of 4MC23O could be prevented by some chemicals other than substrate [21]. As mentioned above, organic solvents such as acetone, propanol, and ethanol activated the 4MC23O (Fig. 3). Enzymes were first

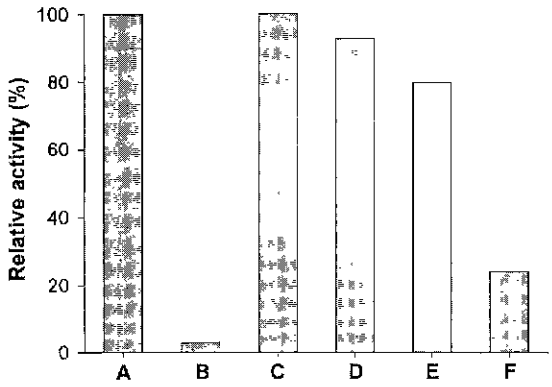

Fig. 5. Protective effect of organic solvents on the inactivation of 4MC23O.

The incubation mixtures contained 50 mM phosphate buffer (pH 7.5), 20 μM of enzyme, 30 μM of H_2O_2, and protecting solvent. Incubations were carried out at 25°C for 30 min. **A** indicates control without inactivator and organic solvent and **B** represents inactivation of enzyme without organic solvent. Organic solvents added were: **C**, 10% acetone; **D**, 10% propanol; **E**, 10% ethanol; **F** 10% methanol.

incubated in the oxidizing agent with 10% of organic solvent, and then the enzyme activities were determined (Fig. 5). The acetone protected the enzyme from the inactivation by hydrogen peroxide. In addition, propanol and ethanol also prevented the denaturation of enzyme. Methanol, which was poor for activating the enzyme (Fig. 3), was also poor for preventing the enzyme from inactivation (Fig. 5). Unfortunately the mechanism of protective action of organic solvent is still not fully understood. Nevertheless, organic solvents must prevent hydrogen peroxide from binding to the ferrous ion in the active site, by making a nonpolar diffusion barrier between H_2O_2 and enzyme. In a case where high concentration of organic solvents exists (over 50%), organic solvent can directly unfold the protein and, consequently, the effect of protection was not observed in this study.

Reactivation Studies of Inactivated 4MC23O

It was reported that inactivated catechol dioxygenases were reactivated by the reducing agents such as ascorbic acid, cysteine, or ferrous ion [21]. We also investigated the reactivation of 4MC23O which was purified from *Pseudomonas putida* SU10, as shown in Table 2. Native enzyme showed 283 units of activity under the standard conditions. Addition of a reducing agent and ferrous ion alone to the native enzyme did not show any activation effect. Instead, ascorbate was clearly shown to decrease the activity. On the other hand, addition of a reducing agent such as ferrous ion doubled the activity by as much as 600 units.

After inactivating the enzyme with 3 μM of hydrogen peroxide, the enzyme exhibited only 30 units of activity. This enzyme was reactivated to half the level of the native enzyme with an individual reducing agent. Incubation with

Table 2. Activation and reactivation of 4-methylcatechol 2,3-dioxygenase by some chemicals.

Chemical treatment for reactivation[1]	Enzyme activity (units)[2]	
	Native enzyme[3]	Inactivated enzyme[4]
None	283	30
Ascorbate (1 mM)	199	120
Cysteine (1 mM)	283	136
Fe^{2+} (1 mM)	340	273
Ascorbate+Fe^{2+} (1 mM of each)	608	512
Cysteine+Fe^{2+} (1 mM of each)	638	635

[1]Chemical treatment conditions: 1 mM of each chemical alone or combined was treated at 25°C for 20 min.

[2]Enzyme activity was determined under the standard conditions: 50 mM of phosphate buffer (pH 7.5); 200 μM of 4-methylcatechol; 35°C and 10 min reaction. One unit of 4MC23O activity is the amount of enzyme that produces 1 μM of HMOH-dienoic acid per min.

[3]Native state is the purified enzyme (20 μM) of 4MC23O without treatment with H_2O_2.

[4]The enzyme (20 μM) treated with 3 μM H_2O_2 for 20 min at 25°C was used as an inactivated state.

ferrous ion alone recovered the activity to almost the same level of untreated enzyme. In addition, the enzyme activities were recovered and activated about 2 times when it was incubated with both reducing agent and ferrous ion. The same phenomenon was observed in the case of metapyrocatechase which was purified from *Pseudomonas arvilla* [21] and catechol dioxygenase (C23O) from *Pseudomonas putida* [3]. The reducing agent with ferrous ion accelerated the activation of enzymes by more than 2-folds. The addition of ferrous ion by itself was effective, whereas reducing agent alone brought about partial reactivation only (Table 2). These results supported the contention that the inactivation of the enzyme by H_2O_2 was mainly due to oxidation of ferrous ion to the ferric form in the active site of the protein. Other reports [1, 2] also support similar conclusion that catechol dioxygenase was fully reactivated and the activity was further accelerated about 6 or 7 times when it was incubated with ferrous ions and reducing agents.

o-Phenanthroline, which was known to be a good nitrogen base for protecting the metapyrocatechase [21], also prevented the 4MC23O from reactivation, maintaining the activity at around 60%. Reactivation of inactivated 4MC23O with cysteine and ferrous ion was prevented when *o*-phenanthroline was present in the reaction mixture (Fig. 6). In the absence of *o*-phenanthroline, most of the enzymes were recovered only after 20 min of incubation, whereas reactivation was markedly prevented in the presence of nitrogen base. Nitrogen bases are well known for their properties of protecting the enzyme competitively, inhibiting the enzyme, and preventing reactivation [7, 22]. In addition, this base bound to the enzyme with high

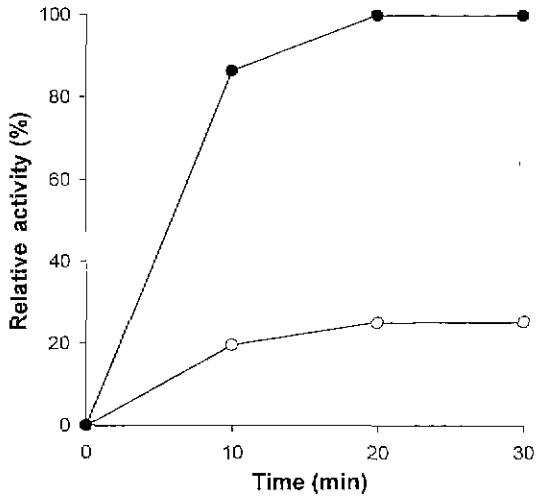

Fig. 6. Effect of *o*-phenanthroline on the reactivation of 4MC23O inactivated by H_2O_2.

The reaction mixture contained 1 mM cysteine and 1 mM ferrous ammonium sulfate in 50 mM phosphate buffer (pH 7.5). Reactivation of inactivated enzyme (20 mM) by H_2O_2 (30 mM) was performed in the presence (●) and in the absence (○) of 1 mM *o*-phenanthroline

affinity and specificity implying higher protective effects, and were also more effective in preventing the reactivation of enzyme. As with the organic solvents, phenanthroline could not reactivate the inactivated enzyme, although it protected the enzyme from denaturation.

Acknowledgments

This work was supported by grants from the Ministry of Science and Technology (KISTEP; 99-N6-02-01-B-03).

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