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Inhibition and Chemical Mechanism of Protocatechuate 3,4-dioxygenase from *Pseudomonas pseudoalcaligenes* KF707

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We carried out pH stability, chemical inhibition, chemical modification, and pH-dependent kinetic parameter assessments to further characterize protocatechuate 3,4-dioxygenase from *Pseudomonas pseudoalcaligenes* KF707. Protocatechuate 3,4-dioxygenase was stable in the pH range of 4.5~10.5. L-ascorbate and glutathione were competitive inhibitors with K_{is} values of 0.17 mM and 0.86 mM, respectively. DL-dithiothreitol was a noncompetitive inhibitor with a K_{is} value of 1.57 mM and a K_{ii} value of 8.08 mM. Potassium cyanide, *p*-hydroxybenzoate, and sodium azide showed a noncompetitive inhibition pattern with K_{is} values of 55.7 mM, 0.22 mM, and 15.64 mM, and K_{ii} values of 94.1 mM, 8.08 mM, and 662.64 mM, respectively. $FeCl_2$ was the best competitive inhibitor with a K_{is} value of 29 μM. $FeCl_3$, $MnCl_2$, $CoCl_2$, and $AlCl_3$ were also competitive inhibitors with K_{is} values of 1.21 mM, 0.85 mM, 3.98 mM, and 0.21 mM, respectively. Other metal ions showed noncompetitive inhibition patterns. The pH-dependent kinetic parameter data showed that there may be at least two catalytic groups with pK values of 6.2 and 9.4 and two binding groups with pK values of 5.5 and 9.0. Lysine, cysteine, tyrosine, carboxyl, and histidine were modified by their own specific chemical modifiers, indicating that they are involved in substrate binding and catalysis.

Key words : Chemical modification, pH-dependent kinetic parameters, protocatechuate 3,4-dioxygenase, *Pseudomonas pseudoalcaligenes* KF707

Introduction

Polyphenol oxidase (monophenol, dihydroxy-L-phenylalanine: oxygen oxidoreductase; EC 1.14.8.1) is a copper-containing enzyme widely distributed in nature and responsible for browning in fruits and vegetables. Polyphenol oxidase is a mixed-function oxidase that catalyzes both the ortho hydroxylation of monophenols to *o*-diphenols (cresolase activity) and the further oxidation of *o*-diphenols to *o*-quinones (catecholase activity). The *o*-quinones are highly reactive compounds that polymerize spontaneously to form brown pigment (melanin) or react with amino acids and proteins [34]. Enzymatic browning is a significant problem during processing of raw agro-materials such as banana, pear, grape, peach, apple, mushroom, potato, lettuce, shrimps, lobster, and crab [12, 23]. Therefore, development of high performance tyrosinase inhibitors is important to prevent browning of vegetables and fruits. Many methods to prevent the browning reaction have been proposed [1], including high temperature [22], sulfur compounds such as sodium sulfite, sodium bisulfite, and sodium metabisulfite [19], acids such as citric acid, malic acid, phosphoric acid, and ascorbic acid [14, 28], reducing agents such as L-cysteine or DL-methionine [24], and boric acid and borates [2]. Although numerous studies have reported attempts to control various aspects of browning reactions by phenolase, little attention has been paid to reactions that can modify its phenolic substrates so that they become unavailable for the darkening reactions. Bacterial oxygenase, which carries out oxidative ring-opening reactions, cleaves the catechol ring of plant-darkening constituents [16]. Use of such ring-opening reactions is a simple method for controlling phenolase darkening of compounds. In a previous study [30], we reported the purification and characterization of protocatechuate 3,4-dioxygenase from *Pseudomonas pseudoalcaligenes* KF707 as a possible anti-browning agent. Here, we describe the inhibition mode of chemicals and the chemical mechanism by evaluating chemical modification and pH-dependent kinetic

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parameters.

Materials and Methods

Materials

MES (2-(N-morpholino)-ethanesulfonic acid), Tris (Tris (hydroxymethyl)-aminomethane), CHES ((2-N-cyclohexyl-amino)-ethanesulfonic acid), and CAPS (3-(cyclohexylamino)-1-propanesulfonicacid) were obtained from Research Organics Inc. (Cleveland, OH, USA). Pyridoxal-5′-phosphate (PLP), 2,4-dinitrofluorobenzoic acid (FDNB), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), ninhydrin, 2,3-butanedione, N-acetylimidazole, iodoacetamide, (1-ethyl-3-(3-dimithylaminopropyl)-carbodiimide) (EDAC), diethylpyrocarbonate (DEPC), and *o*-methylisourea were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade and used without further purification. All solutions were made using distilled water deionized with a 0.22 μm filter and a Mili-Q plus reagent water system (Millipore Co., Molsheim, France).

Protocatechuate 3,4-dioxygenase assay

All assays were carried out using a CARY 3 Bio Spectrophotometer (Varian Australia Pty. Ltd., Melbourne, Australia). Kinetic parameters of protocatechuate 3,4-dioxygenase was determined as described previously [30]. The assay mixture contained 50 mM air-saturated buffer (Tris, pH 7.5) and 48 μM protocatechuate in the total volume of a 1 mL quartz cuvette (1 cm width). The reaction was initiated by adding 10 μl enzyme solution (1 unit) with an adder-mixer. One unit of enzyme activity was defined as the amount of enzyme that converts 1 mmol of substrate per minute. Enzymatic activity was determined with 3,4-dihydroxyphenylacetate at 258 nm (ε = 9.4 $mM^{-1}cm^{-1}$) for the conversion of protocatechuic acid to 3-carboxy-cis,cis-muconic acid [13].

Protein determination

Protein concentration was determined with the Bio-Rad Protein Assay Kit (Hercules, CA, USA), according to the Bradford method. Crystalline bovine serum albumin (BSA) was used as the standard, as described previously [3].

pH stability

Buffers were chosen for adequate buffer capacity, and more than one buffer was used to examine the effect of buffer on activity. Buffers at a 50 mM final concentration were used over the following pH ranges: Acetate, pH 4.5~5.5; MES, pH 5.5~6.5; phosphate, pH 6.5~7.5; Tris, 7.5~8.8; CHES, pH 8.8~10; and CAPS, pH 10~10.5. The pH of the reaction mixtures was measured before and after sufficient data were collected to determine initial velocities. Negligible pH changes were observed before and after the reaction. A concentrated enzyme solution (10 units) was mixed with 980 μl of each 50 mM buffer. An aliquot was taken out at different times (every 5 to 10 minutes) and assayed for remaining activity at 25°C. Protocatechuate 3,4-dioxygenase activity was measured with 48 μM protocatechuate in a 50 mM air-saturated buffer (Tris, pH 7.5). The pH of the reaction mixtures were checked before and after the reaction. All assays were performed in duplicate.

pH studies

All buffers were titrated to the desired pH with KOH. In all cases, overlaps were obtained when buffers were changed so that a correction could be made for spurious buffer effects. Negligible pH changes were observed before and after the reaction. All assays were performed in duplicate.

Effect of inhibitors

Organic (L-cysteine, glutathione, L-ascorbate, β-mercaptoethanol, DL-dithiothreitol, and *p*-hydroxybenzoic acid) and inorganic (sodium azide, potassium cyanide, thiourea, $FeCl_2$, $AgNO_3$, $CuCl_2$, $FeCl_2$, $ZnCl_2$, $SnCl_2$, $AlCl_2$, $MnCl_2$, $CoCl_2$, $NiCl_2$, $MgSO_4$, KCl, NaCl, and $CaCl_2$) compounds were used for the inhibition study. The materials selected for the inhibition study were added to a reaction mixture (1 mL) containing 50 μM protocatechuate and 50 mM Tris buffer (pH 7.5). The reaction was initiated by adding 10 μl enzyme solution (1 unit) and monitored at 258 nm. All assays were performed in duplicate.

Chemical modification

Enzyme solution (10 unit) was incubated with different concentrations of chemical modifiers in 100 μl of 50 mM Tris buffer (pH 7.5), as described previously [4]. The chemical modifiers used in this study were PLP, FDNB, DTNB, ninhydrin, 2,3-butanedione, N-acetylimidazole, iodoacetamide, EDAC (1-ethyl-3-(3-dimithylaminopropyl)-carbodiimide), diethylpyrocarbonate, and *o*-methylisourea. A 20 μl aliquot was withdrawn at specified time intervals and assayed for

enzyme activity. All assays were performed in duplicate.

Data processing

All kinetic data were fitted using the appropriate rate equations [5]. Data for initial velocity studies were fit to Eq. 1. Data for competitive, noncompetitive, uncompetitive inhibition were fit to Eq. 2-3, respectively. In Eq. 1-4,

$$v = V_{max}A/[K_m + A] \quad (1)$$

$$v = V_{max}A/[K_m (1+I/K_{is})+A] \quad (2)$$

$$v = V_{max}A//[K_m (1+I/K_{is})+A(1+I/K_{ii})] \quad (3)$$

where v is observed velocity; V_{max} is maximum velocity; A is substrate concentration; I is inhibitor concentration; K_m is the Michaelis constant; K_{is} is the slope of the inhibition constant; and K_{ii} is the intercept of the inhibition constant. In the pH studies, the individual saturation curves used to obtain pH profiles were fit to Eq. 1. Data for the pH profiles that decreased with slopes of +1 and -1 were fit using Eq. 4.

$$\log Y = \log C/[1+(H/K_1)+(K_3/H)] \quad (4)$$

where A and H are the reactant and hydrogen ion concentrations, respectively. The constant K_1 represents the acid dissociation constant for the enzyme or reactant functional groups reflected on the acid side of the pH profiles; K_3 represents the acid dissociation constant for the enzyme or reactant functional groups reflected on the basic side of the pH profiles. Y is the value of the parameter observed as a function of pH, and C is the pH-independent value of Y.

Results and Discussion

Antioxidant and chemical inhibitory modes

Kinetic data obtained using different concentrations of L-ascorbate, DL-dithiothreitol, glutathione, potassium cyanide, *p*-hydroxybenzoate, and sodium azide were subjected to a double-reciprocal plots analysis. To establish the type of inhibition and to determine the kinetic parameters K_{is} and K_{ii}, the kinetic program of Cleland [5] was used, and the results are given in Table 1. L-ascorbate and glutathione were competitive inhibitors with K_{is} values of 0.17 mM and 0.86 mM, respectively. DL-dithiothreitol was a noncompetitive inhibitor with a K_{is} value of 1.57 mM and a K_{ii} of 8.08 mM. Durham et al. [8] reported that glutathione and dithiothreitol do not inhibit protocatechuate 3,4-dioxygenase from *Azotobacter vinelandii*. L-Cysteine does not act as an inhibitor of protocatechuate 3,4-dioxygenase, whereas it is an inhibitor of phenolase [15]. Potassium cyanide, *p*-hydroxybenzoate, and sodium azide were noncompetitive inhibitors with K_{is} values of 55.7 mM, 0.22 mM, and 15.64 mM, and K_{ii} values of 94.1 mM, 8.08 mM, and 662.64 mM, respectively.

Table 1. Inhibition parameters of antioxidants and chemicals for the protocatechuate 3,4-dioxygenase

Inhibitor	Inhibition Pattern	Inhibition Constants	
		Kis (mM)	Kii (mM)
L-Ascorbic acid	C	0.17	-
DL-Dithiothreitol	NC	1.57	9.28
Glutachione	C	0.86	-
P-Hydroxybenzoate	NC	0.22	9.28
Sodium azide	NC	15.64	662.4
Potassium cyanide	NC	55.7	94.1

C: competitive inhibition, NC: noncompetitive inhibition, Kis: slope inhibition constant, Kii: intercept inhibition constant.

Metal ion inhibitory modes

$FeCl_2$, $FeCl_3$, $MnCl_2$, $CoCl_2$, $AgNO_3$, $CaCl_2$, $AlCl_3$, $MgSO_4$, $CuCl_2$, $ZnCl_2$, $SnCl_2$, $NiCl_2$, KCl, and NaCl were used to investigate their effects on protocatechuate 3,4-dioxygenase activity from *Pseudomonas pseudoalcaligenes* KF707, and the results are shown in Table 2. Most metal ions showed inhibitory activity. $FeCl_2$ was the best competitive inhibitor with a K_{is} value of 29 μM. $FeCl_3$, $MnCl_2$, $CoCl_2$, and $AlCl_3$ were also competitive inhibitors with K_{is} values of 1.21 mM, 0.85 mM, 3.98 mM, and 0.21 mM, respectively. Ferrous ion

Table 2. Inhibition parameters of metal ions for the protocatechuate 3,4-dioxygenase

Inhibitor	Inhibition Pattern	Inhibition Constants	
		Kis (mM)	Kii (mM)
$FeCl_2$	C	2.9×10^{-2}	-
$FeCl_3$	C	1.21	-
$MnCl_2$	C	0.82	-
$CoCl_2$	C	3.98	-
$AlCl_3$	C	0.21	-
$AgNO_3$	NC	3×10^{-8}	1.6×10^{-7}
$CaCl_2$	NC	8.9×10^{-2}	7.6×10^{-2}
$ZnCl_2$	NC	0.65	0.14
$SnCl_2$	NC	1.52	0.34
$NiCl_2$	NC	4.5×10^{-2}	0.34
KCl	NC	109	566
NaCl	NC	21	193
$MgSO_4$	NC	0.17	5.36

C: competitive inhibition, NC: noncompetitive inhibition, Kis: slope inhibition constant, Kii: intercept inhibition constant.

had a low K_{is} value of 29 μM, indicating that the active site ferric ion is not tightly bound in this enzyme. Kurahashi et al. [17] reported that the X-ray crystal structure of protocatechuate 3,4-dioxygenase from *Pseudomonas putida* has a trigonal-bipyramidal ferric ion center with four endogenous protein ligands of Tyr408, Tyr447, His460, and His462 and a solvent-derived ligand. However, no reports are available on the ferrous ion inhibition of protocatechuate 3,4-dioxygenase from *Pseudomonas putida*. Dagley [6] reported that anions such as phosphate, acetate, and chloride are competitive inhibitors of the protocatechuate 3,4-dioxygenase from *Pseudomonas aeruginosa*. $AgNO_3$, $CaCl_2$, $ZnCl_2$, $NiCl_2$, KCl, NaCl, and $MgSO_4$ are noncompetitive inhibitors. $AgNO_3$ was the best noncompetitive inhibitor with a K_{is} value of 0.03 pM and a K_{ii} of 0.16 pM. The K_{is} and K_{ii} values for other metal ions were as follows: 89 μM and 76 μM for $CaCl_2$, 0.65 mM and 0.14 mM for $ZnCl_2$, 1.52 mM and 0.34 mM for $SnCl_2$, 45 μM and 0.34 mM for $NiCl_2$, 109 mM and 566 mM for KCl, 21 mM, and 193 mM for NaCl, 0.17 mM and 5.36 mM for $MgSO_4$, respectively.

pH stability

The pH stability profile is shown in Fig. 1, indicating that the enzyme was stable in the pH range of 4.5~10.5. This means that there was only a change in ionization state of functional amino acids for substrate binding and catalysis, not in the three-dimensional structure of the enzyme.

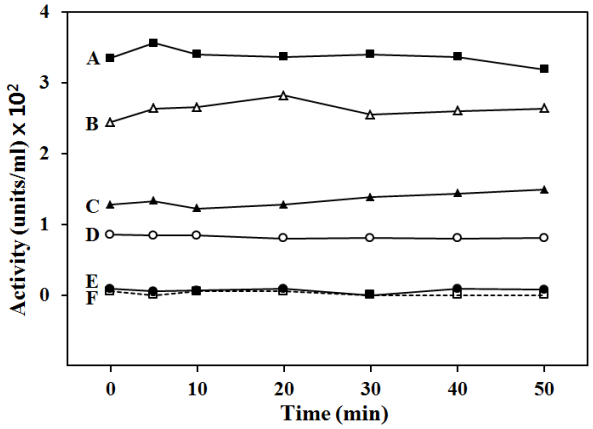

Fig. 1. pH Stability of the protocatechuate 3,4-dioxygenase. A concentrated enzyme solution (10 units) was mixed with 980 μl of each 50 mM buffer. An aliquot was taken out at different times and assayed for remaining activity at 25°C. Protocatechuate 3,4-dioxygenase activity was measured with 48 μM protocatechuate in a 50 mM air-saturated buffer (Tris, pH 7.5). (A) pH 7.0, (B) pH 8.5, (C) pH 6.4, (D) pH 5.7, (E) pH 4.6, (F) pH 10.5

pH profiles of the kinetic parameters

Changes in V_{max}, and $V_{max/Km}$ values depend on ionization of both the free enzyme and the enzyme-substrate complex [10, 29]. Protocatechuate 3,4-dioxygenase from *Pseudomonas pseudoalcaligenes* KF707 was stable over the pH range 4.5~10.5 for at least 50 min, as mentioned above. The pH profiles for the V_{max} and $V_{max/Km}$ values are shown in Fig. 2, respectively. The V_{max} values decreased at low and high pHs with slopes of + 1 and - 1, respectively. Fitting Eq. 4 to the data provided pK values of 6.2 and 9.4 on the acidic and basic sides, respectively. The V_{max}/K_m values also decreased at low and high pHs with slope of + 1 and - 1, respectively. Fitting Eq. 4 to the data provided pK values of 5.5 and 9.0 on the acidic and basic sides, respectively. It was assumed that there are at least two catalytic groups with pK values of 6.2 and 9.4 and two binding groups with pK values of 5.5 and 9.0 based on the result that the V_{max} profile exhibited pK values for the catalytic group and V_{max}/K_m profile for binding and catalytic groups of enzyme and reactants. However, no reports on pH profiles of the kinetic parameters for protocatechuate 3,4-dioxygenases are available.

Chemical modification of protocatechuate 3,4-dioxygenase

To confirm the amino acid residues that were proposed to be involved in binding and catalysis at the active site of

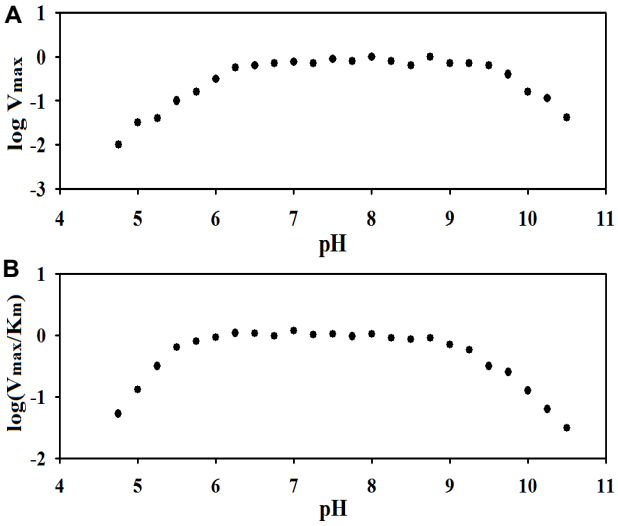

Fig. 2. pH-Dependent kinetic parameters of the protocatechuate 3,4-dioxygenase. Kinetic parameters were measured in a 50 mM air-saturated buffer at different pHs. Points are experimental values obtained from the double reciprocal plots of each pH. (A)log (Vmax) versus pH, (B)log (Vmax/Km) versus pH.

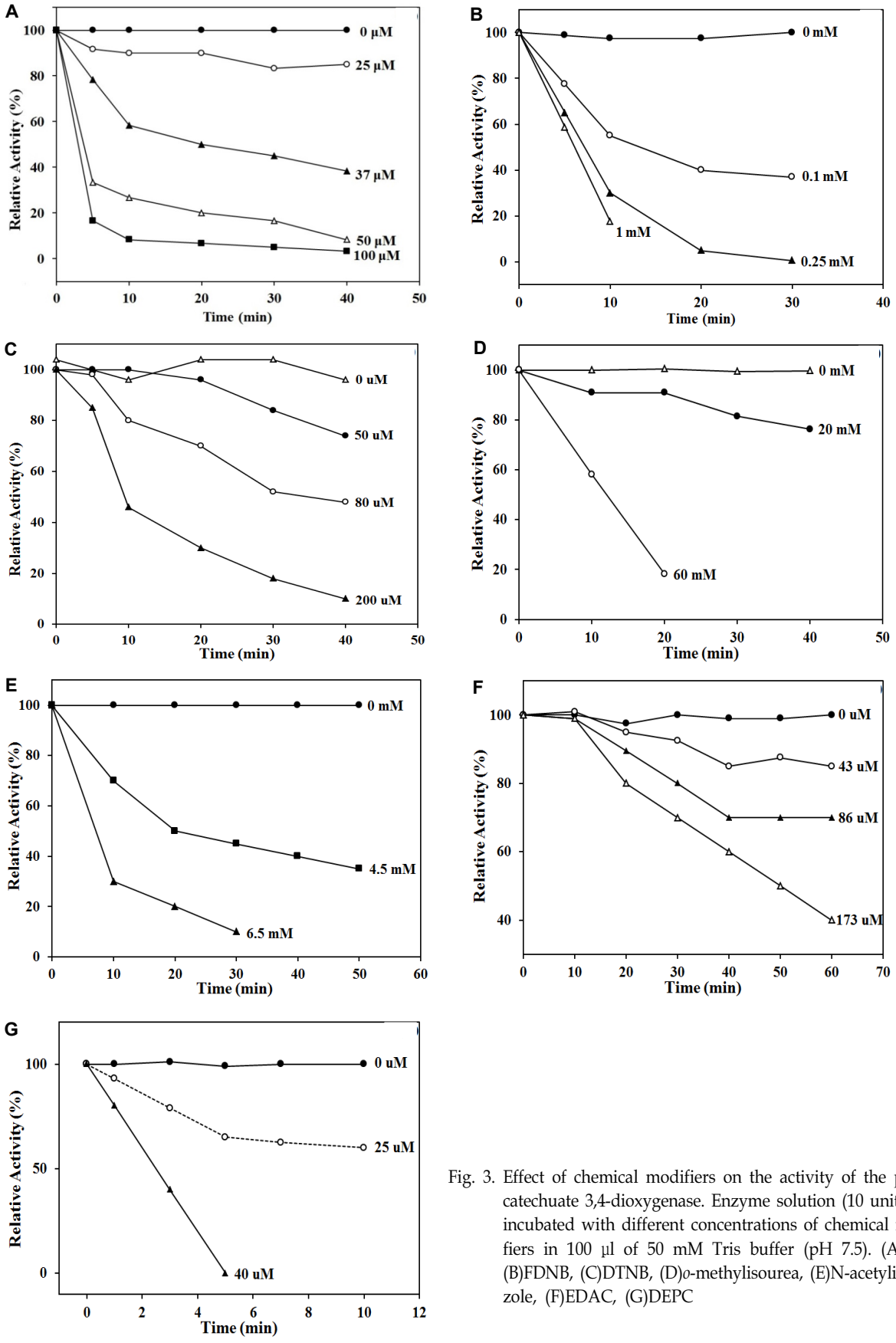

Fig. 3. Effect of chemical modifiers on the activity of the protocatechuate 3,4-dioxygenase. Enzyme solution (10 unit) was incubated with different concentrations of chemical modifiers in 100 μl of 50 mM Tris buffer (pH 7.5). (A)PLP, (B)FDNB, (C)DTNB, (D)*o*-methylisourea, (E)N-acetylimidazole, (F)EDAC, (G)DEPC

the enzyme, the chemical modifiers: PLP and FDNB, DTNB and *o*-methylisourea, ninhydrin and 2,3-butanedione, N-acetylimidazole, 1-ethyl-3-(3-dimethylaminoptopyl)-carbodiimide (EDAC), diethylpyrocarbonate, and iodoacetamide were used to modify the lysine, cysteine, arginine, tyrosine, carboxylate, histidine, and methionine side chains, respectively [20, 21]. PLP inactivated the enzyme in a biphasic pattern with 100% inactivation at > 50 μM. FDNB also inactivated the enzyme in a biphasic pattern with 100% inactivation at > 250 μM. These data strongly suggest that a lysine residue is involved in binding and/or catalysis. DTNB and *o*-methylisourea inactivated the enzyme in a linear pattern with 100% inactivation at > 0.2 mM and 60 mM, respectively. This result suggests that a cysteine residue plays a role as a binding or catalytic amino acid. However, ninhydrin and 2,3-butanedione did not inhibit enzyme activity, indicating that the arginine residue is absent at the active site. N-acetylimidazole inactivated the enzyme in a biphasic pattern with 100% inactivation at > 6.5 mM, suggesting that a tyrosine residue is present at the active site. Chemical modification by EDAC resulted in a linear time-dependent inactivation pattern, indicating that carboxylate may be involved in the binding or catalytic step. Diethylpyrocarbonate inactivated the enzyme in a linear pattern with 100% inactivation at > 40 μM, suggesting that a histidine residue is present at the active site. However, the enzyme was not inactivated by iodoacetamide, indicating that methionine is not a part of the active site. All data are shown in Fig. 3. Ohlendorf et al. [25] reported that tyrosine and histidine side chains exist at the active site by X-ray crystallography. Thus, tyrosine and histidine residues are present in both enzymes. Taken together, the chemical modification data show that the side chains of cysteine, tyrosine, histidine, carboxylate, and lysine exist at the active site of protocatechuate 3,4-dioxygenase from *Pseudomonas pseudoalcaligenes* KF707.

Chemical mechanism

The pH profiles of the kinetic parameters show that at least two amino acid groups with pK_r values of 6.2 and 9.4 for catalysis and at least two amino acid groups with pK_r values of 5.5 and 9.0 for binding exist at the active site. The chemical modification data show that the side chains of tyrosine, histidine, cysteine, and lysine are at the protocatechuate 3,4-diooygenase active site. The combination of data from the pH study and chemical modification suggest that cysteine (pK_r 6.2) and tyrosine (pK_r 9.4) serve as catalytic groups, and that histidine (pK_r 5.5) and lysine (pK_r 9.0) serve as binding groups. The possible reason for the lower pK values for each side chain is that deprotonation may occur much faster in the hydrophobic condition. For example, Lim et al. [18] reported that the pK_r of the active site cysteine of mouse methionine sulfoxide reductase is 7.2 even in the absence of substrate. However, carboxylate was not shown up in the pH titration. It is carefully assumed that too low a carboxylate pK_r value could not be reflected in the tested pH range. Protocatechuate 3,4-dioxygenases isolated from *Acinetobacter calcoaceticus* ADP1, *Brevibacterium fuscum* [31], and *Pseudomonas putida* [32] are the best characterized structurally. Many X-ray crystallographic structures are available for protocatechuate 3,4-dioxygenases from *Pseudomonas putida* and *Acinetobacter calcoaceticus* ADP1 [9, 11, 25-27, 33]. The data show that the geometry of the high-spin ferric center is in a trigonal bipyramidal arrangement around the metal with histidine and tyrosinate as axial ligands and histidine, tyrosinate, and solvent-derived OH groups as equatorial ligands [25]. X-ray absorption spectroscopy of 3,4-PCD from *Brevibacterium fuscum* and *Pseudomonas putida* reveal that the water-based ligand is a hydroxide and thus charge neutrality of the active site was obtained [31, 7]. However, these studies show that histidine and tyrosine residues are involved in binding and catalysis and not cysteine and lysine. Based on the finding that cysteine, lysine, histidine, carboxylate, and tyrosine are involved in binding or catalysis of protocatechuate 3,4-dioxygenase from *Pseudomonas pseudoalcaligenes* KF707, we propose the possible active site map and chemical mechanism as shown in Fig. 4 and 5.

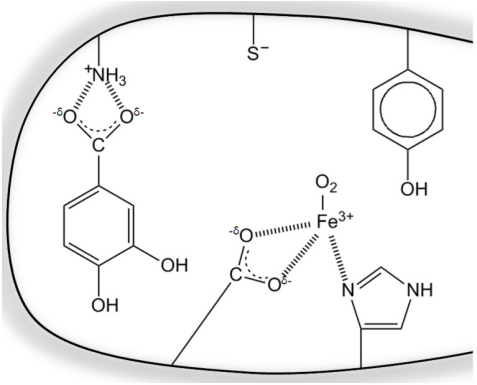

Fig. 4. Proposed active site map of the protocatechuate 3,4-dioxygenase. The data show that cysteine, lysine, histidine, carboxylate, and tyrosine are involved in binding or catalysis of protocatechuate 3,4-dioxygenase from *Pseudomonas pseudoalcaligenes* KF707.

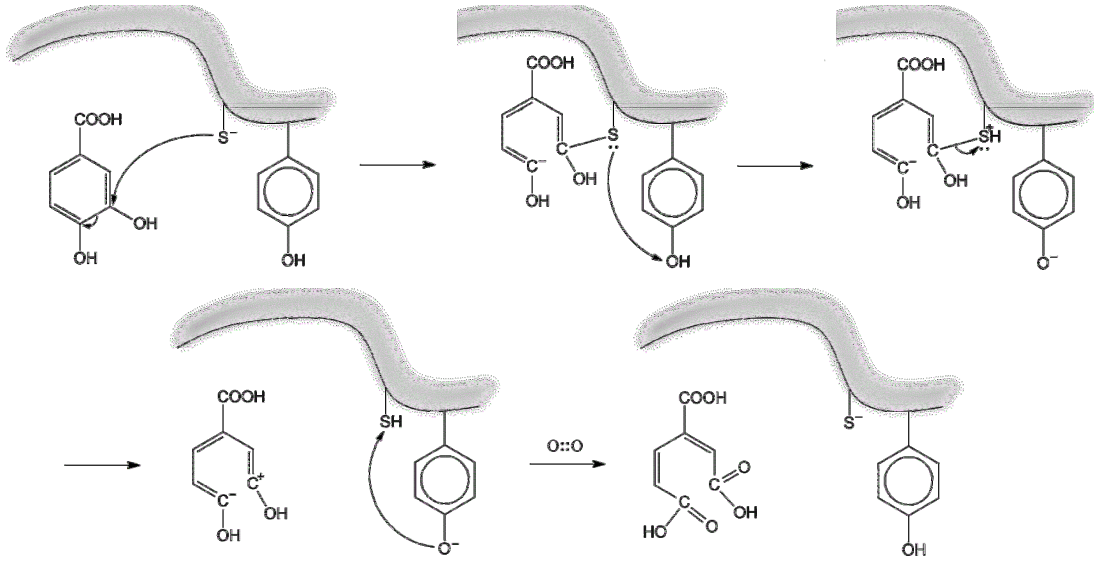

Fig. 5. Proposed chemical mechanism of the protocatechuate 3,4-dioxygenase. Catalysis occurs by binding of Fe^{3+} to the imidazole ring of histidine and carboxylate oxygen ($O\delta^-$), and subsequent binding of O_2 to Fe^{3+}, and the carboxylate of protocatechuate to the lysine residue.

Catalysis occurs by binding of Fe^{3+} to the imidazole ring of histidine and carboxylate oxygen ($O\delta^-$), and subsequent binding of O_2 to Fe^{3+}, and the carboxylate of protocatechuate to the lysine residue. Then, the deprotonated sulfhydryl attacks C_3 of protocatechuate with subsequent cleavage of the C_3 - C_4 bond. C_4 obtains an electron from C_3 and becomes C_4^- by subsequent cleavage of the C_3 - C_4 bond. A sulfur electron attacks the hydroxyl proton of tyrosine and the C_3 - S bond is broken with C_3^+ remaining. Oxygen dissociates into negative and positive oxygen elements and binds to C_3^+ and C_4^-, respectively, with two resulting carboxylates. In contrast, the protonated sulfhydryl is attacked by a deprotonated hydroxyl of tyrosine and returns to its original state for another catalysis reaction.

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초록 : *Pseudomonas pseudoalcaligenes* KF707에서 유래한 protocatechuate 3,4-dioxygenase의 저해 및 화학적 메커니즘

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Pseudomonas pseudoalcaligenes KF707에서 정제한 protocatechuate 3,4-dioxygenase의 특징을 조사하기 위하여 pH안정성, 화학적 저해, 화학적 수식과 pH의존성 반응 상수에 대한 실험을 수행하였다. 이 효소는 pH 4.5~10.7에서 안정하였다. L-ascorbate와 glutathione은 Kis가 각각 0.17 mM과 0.86 mM인 경쟁적 저해제였으며, DL-dithiothreitol은 Kis 1.57 mM 및 Kii 8.08 mM의 비경쟁적 저해패턴을 나타내었다. Potassium cyanide, *p*-hydroxybenzoate 및 sodium azide는 Kis가 각각 55.7 mM, 0.22 mM 및15.64 mM이었으며, Kii는 각각94.1 mM, 8.08 mM, 및 662.64 mM인 비경쟁적 저해패턴을 나타내었다. $FeCl_2$는 Kis가 29 μM로 가장 우수한 경쟁적 저해제였으며, $FeCl_3$, $MnCl_2$, $CoCl_2$, $HgCl_2$, $AlCl_3$도 각각 Kis가 1.21 mM, 0.85 mM, 3.98 mM, 0.17 mM 및 0.21 mM인 경쟁적 저해패턴을 보였다. 한편, 다른 금속이온들은 비경쟁적 저해패턴을 나타내었다. pH의존성 반응상수의 실험결과로부터 pK 6.2와 9.4의 촉매부위와 pK 5.5와 9.0의 결합부위가 존재함을 알 수 있었다. Lysine, cysteine, tyrosine, carboxyl과 histidine은 각각의 고유한 화학적 수식제에 의해 수식되었는데, 이는 이들 잔기들이 결합과 촉매에 관여한다는 것을 나타낸다. 위 결과를 토대로 화학적 메커니즘을 제시한다.