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ORIGINAL ARTICLE

Purification and Characterization of Protocatechuate 3,4-dioxygenase from *Pseudomonas pseudoalcaligenes* KF707

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Abstract Protocatechuate 3,4-dioxygenase was isolated and characterized from *Pseudomonas pseudoalcaligenes* KF707 for the purpose of developing a new anti-browning agent. The protocatechuate 3,4-dioxygenase from *Pseudomonas pseudoalcaligenes* KF707 was purified 296.8-fold, and showed specific activity of 121.7 U/mg. Based on the SDS-polyacrylamide and gel permeation chromatography, the molecular weight of protocatechuate 3,4-dioxygenase was 189.9 kDa and was composed of 3 αβ protomers, with molecular weights of 29.0 kDa of α subunit and 34.3 kDa of â subunit. The optimal pH and temperature were 7.5 and 38°C, respectively. K_m values of catechol, protocatechuate, gallate, *p*-cresol, caffeic acid, catechin, L-DOPA, 4-methylcatechol and pyrogallol were 14, 17, 2, 10, 12, 20, 30, 21 and 3 μM, and the V_{max}/K_m (mim^{-1}) values were 0.052, 3.06, 0.35, 0.01, 0.03, 0.02, 0.006, 0.008 and 0.11, respectively. This indicates that the enzyme is active on a wide range of phenyl compounds, in contrast to the high specificity of similar enzymes from other sources. Our data also show that the turnover number of protocatechuate 3,4-dioxygenase from *Pseudomonas pseudoalcaligenes* KF707 is 68 s^{-1}, which is much higher than the known values from other sources.

Introduction

Browning reactions in foods are popular phenomena because browning precursors are present in foods. Some known browning precursors and their associated foods include L-3-(3,4-dihydroxyphenyl)alanine (apple, pear, plum and grape), chlorogenic acid (apple, pear, peach, sweet cherry, plum, and grape), caffeic acid (apple, pear, apricot, peach, apricot, and grape), catechol (apple, pear, apricot, and grape), (+)-catechin (grape, plum, apricot, pear, apple, and peach), protocatechuic acid (pear and grape), pyrogallol (peach), *p*-cresol (apple and pear), (-)-epicatechin(apple), 4-methylcatechol (apple, apricot, and sweet cheery), gallic acid (peach and grape), 3,4-dihydroxtphenethylamine (peach), as well as resorcinol, hydroquinone, phenol and 3,4-dihydroxypropiophenone (Vamos-Vigyazo, 1981). Browning reactions contribute to the development of desirable flavors and colors of food products such as coffee, beer, raisins and toasted bread. However, browning reactions may also be undesirable due to causation of off-flavors and poor appearance in many foods, such as vegetables and fruits, as well as frozen and dehydrated foods. Another significant problem with browning is the lowering of the nutritive value of the food and the reduction of shelf-life.

Although numerous studies have attempted 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 may become unavailable to darkening reactions. Methylation of phenolase substrate was performed using a meta-*O*-methyltransferase existing in plant tissues (Finkle and Nelson, 1963). This enzyme will function in the presence of a methyl donor, such as *S*-adenosyl methionine, and a methyl acceptor, such as *o*-diphenol. This method makes fruits and vegetables resistant to browning without affecting the original color, flavor, odor or texture (Finkle, 1964). It was also shown that bacterial oxygenate that carries out oxidative ring-opening reactions can cleave the catechol ring of plant-darkening constituents, such as caffeic acid, chlorogenic acid, and dopamine (Kelly and Finkle, 1969). The use of such ring-opening reactions would be a simple method for controlling phenolase darkening of the compounds. It is apparent, however, that the reaction of phenolase-catalyzed

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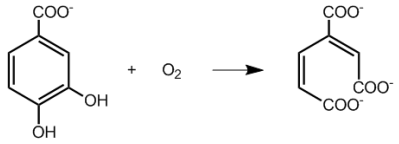
darkening, and this process, along with that of oxygenase-catalyzed ring cleavage, both require oxygen. A primary requirement for controlling oxidative darkening would then be to minimize the oxygen-requiring phenolase reaction while optimizing the oxygen-requiring ring-cleaving oxygenase reaction. The reaction rates of the two oxidative effects can be controlled through the manipulation of the reaction pH and through the use of reducing agents in the reaction solution. Ring-cleaving oxygenase may be developed as an anti-browning agent if the substrate specificity of the enzyme on the browning precursors is low. In this study, protocatechuate 3,4-dioxygenase (EC 1.13.11.3) was chosen as an oxidative ring-opening enzyme for this purpose. Protocatechuate 3,4-dioxygenase, intradiol cleavage dioxygenase, catalyzes the ring-cleavage of protocatechuate to 3-carboxy-cis,cis-muconate as shown below (Fujisawa and Hayaishi, 1968; Broderik and O'Halloran, 1991).



Protocatechuate 3,4-dioxygenase acts as a key enzyme in the biodegradation of aromatic compounds such as protocatechuate, catechol, pyrogallol and so on (Ponting et al., 1971; McCord and Kilara, 1983; Seib, 1985). The aromatic ring of these compounds is opened during reactions catalyzed by dioxygenases, in which both atoms of oxygen from O_2 are incorporated into the substrate. These enzymes usually contain non-heme iron stabilized in either the Fe(II) or Fe(III) oxidation state (Que, 1983; Dagley, 1984). All of the known Fe(III)-containing dioxygenases that function in these pathways utilize catecholic substances. Protocatechuate 3,4-dioxygenase has been isolated and characterized from many bacteria: *Pseudomonas putida* (classified as *Pseudomonas aeruginosa* at the time)(Fujisawa and Hayaishi, 1968; Bull and Ballou, 1981), *Azotobacter vinelandii* (Durham et al., 1980), *Brevibacterium fusum* (Whittaker et al., 1984), *Chaetomium piluliferum* (Wojts-Wasilewska and Trojanowski, 1980), *Acinetobacter calcoaceticus* (Vetting, 1994), *Pseudomonas cepacia* (Ludwig, 1984), *Pseudomonas fluoescens* PHK (Pujar, 1983), *Pseudomonas masrginata* (Peterson, 1996).

In this report, we describe the purification and characterization of protocatechuate 3,4-dioxygenase from *Pseudomonas pseudoalcaligenes* KF707 (Bedard et al., 1986; Taira et al., 1992), which secretes protocatechuate 3,4-dioxygenase strongly, as a new strategy towards the prevention of browning reactions.

Materials and Methods

Materials. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration molecular weight markers were from Boehringer Mannheim (Germany). DNase (deoxyribonuclease), reagents for electrophoresis and Bio-Scale Q2 column/Econo-Pac Methyl hydrophobic interaction chromatography (HIC) cartridge was obtained from Bio-Rad (USA). L-Tyrosine and potassium dihydrogen phosphate were of Junsei Chemical Co., Japan. Yeast extract was from Difco (USA). Mes (2-(N-Morpholino)-ethanesulfonic acid), Tris (Tris(hydroxymethyl)-aminomethane), CHES((2-N-cyclohexylamino)ethanesulfonic acid) and CAPS (3-(cyclohexylamino)-1-propanesulfonicacid) were from Research Organics Inc. (USA). *Pseudomonas pseudoalcaligenes* KF707 was kindly contributed by Professor Kyoung Lee, Department of Microbiology, Changwon National University. All other chemicals and reagents were of analytical grade, and were used without further purification. All solutions were prepared using distilled water filtered through a 0.22 μm filter, from a Mili-Q plus reagent water system (Millipore Co., France).

Bacterial Growth and Cell Extraction. *Pseudomonas pseudoalcaligenes* KF707 was grown at 25°C in "Complex Medium Growth" with 0.5% yeast extract in a 50 mL Erlenmeyer flask, and was used as inoculum of cultures containing 500 mL mineral salts broth (MSG) and 0.1% *p*-hydroxybenzoate as both inducer and carbon source, in a 2 L Erlenmeyer flasks (Stanier et al., 1966). Incubation was carried out in ashaking incubator for 8 h with 150 rpm at 25°C. After the incubation period, the cells were harvested by centrifugation at 10,000×g for 20 min at 4°C.

Assay of Protocatechuate 3,4-Dioxygenase. All assays were carried out using a CARY 3 Bio Spectrophotometer (Varian Australia Ltd., Australia). Protocatechuate 3,4-dioxygenase activity was determined as described previously (Contzen et al., 2000). The assay mixture contained 50 mM air-saturated buffer (Tris, pH 7.5) and 50 μM protocatechuate in a total volume of 1 mL quartz cuvette (1 cm width). The reaction was initiated by the addition of 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 min. The enzymatic activity with 3,4-dihydroxyphenylacetate was determined at 258 nm (ε=9.4 mM^{-1} cm^{-1}) for the conversion of protocatechuic acid to 3-carboxy-cis,cis-muconic acid (Fujisawa et al., 1972).

Purification of Protocatechuate 3,4-Dioxygenase from *Pseudomonas pseudoalcaligenes* KF707. Buffers A, B, C, D and E refer to 50 mM Tris (pH 7.5) containing 1 mM dithiothreitol and 5% glycerol (v/v), 50 mM Tris (pH 7.5) containing 1 mM dithiothreitol, 50 mM Tris (pH 7.5), 50 mM Tris (pH 7.5) containing 1 M NaCl, 50 mM Tris (pH 7.5) containing 0.5 M $(NH_4)_2$ SO_4, respectively. Cell paste (537.6 g) was suspended twice with buffer A containing 0.1 mM PMSF and 1 μg/mL of DNase. The suspension was incubated for 30 min at room temperature. Cell-free extract was prepared by ultrasonication at 150 W for 5 min on ice. 380 mL of the cell-free extract was obtained by centrifugation at 20,000×g for 20 min. 196 g of solid ammonium sulfate was added to the cell-free extract over 2 h with constant stirring for 8 h, after which the resulting suspension was centrifuged for 20 min at 20,000×g. Residual $(NH_4)_2SO_4$ in the pellet were removed by dialysis. The dialyzed solution had added

to it a final 1 mM dithiothreitol and 25% (w/v) glycerol, and was then divided into 500 μL aliquots and stored at −80°C. Protein purification was conducted with a fast performance liquid chromatography (FPLC) system (Bio-Rad, USA). After washing with 40 mL of buffer C, Bio-Scale Q2 column was eluted with 20 mL of buffer D for 5 min after sample injection. Active fractions were eluted with 0.34 M NaCl in buffer D. Fractions were collected at a flow rate of 2 mL/min, dialyzed for 2 h against 500 mL of cold buffer B with 2 changes (250 mL each) and immediately placed in a refrigerator. Dialyzed fractions were chromatographed using Econo-Pac Methyl HIC column (1×5 mL, Bio-Rad). Active fractions were found at 0.35 M $(NH_4)_2SO_4$ of buffer E. Active fractions were pooled, concentrated and stored at −80°C.

Protein Determination. The protein concentration was determined using a Bio-Rad protein assay kit, according to the method of Bradford (1976). Crystalline bovine serum albumin was used as a standard protein. During the chromatographic purification steps, protein concentration in the fractions was followed by reading the absorbance at 280 nm.

Polyacrylamide Gel Electrophoresis. The samples from purification steps were analyzed by discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970) with low molecular weight markers, using the Mini-Protein II Cell apparatus (Bio-Rad, Mississauga, Canada). Commercial low molecular weight calibration kit contained rabbit phosphorylase b (MW 97.5 kDa), bovine serum albumin (MW 66.2 kDa), ovalbumin (MW 43 kDa), carbonic anhydrase (MW 29 kDa), trypsin inhibitor (MW 20.1 kDa) and lysozyme (MW 14.3 kDa). The gel composition was 4% polyacrylamide and 12% polyacrylamide for separating and stacking gels, respectively. Samples were boiled for 5 min for denaturation. To check the homogeneity of the enzyme, polyacrylamide gel electrophoresis was carried out according to the method of Davis (1964) on 10% acrylamide gel without SDS. The gel was stained with Coomassie Brilliant Blue R-250 by boiling for 5 min and was then de-stained in 45% methanol-10% acetic acid overnight. The molecular weight was obtained from the relationship between log(MW) and the relative mobility.

Molecular Weight Determination. For determination of native molecular weight, a Sephadex G-150 column (1.5×100 cm) was equilibrated with 50 mM Tris buffer (pH 7.5). Marker proteins were carbonic anhydrase (MW 29 kDa), ovalbumin (MW 43 kDa), bovine serum albumin (MW 66.2 kDa), rabbit phosphorylase b (MW 97.5 kDa), β-galactosidase (MW 116 kDa) and immunoglobulin G (MW 150 kDa). The molecular weight was obtained from the relationship between log(MW) and elution volume.

Optimum pH. Buffers were chosen for the adequate buffer capacity and more than one buffer was used to examine the effect of buffers on the activity. Buffers at 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 10–10.7. pH of the reaction mixture was measured before and after sufficient data were collected for determining initial velocities. Negligible pH changes were observed before and after the reaction. Protocatechuate 3,4-dioxygenase activity was measured with 50 μM protocatechuate over a pH range of 4.5–10.5. The pH of the reaction mixtures were measured before and after the reaction.

Optimum Temperature. The optimum temperature for enzyme activity was obtained by conducting the assay at various temperatures from 0 to 80°C in 50 mM Tris buffer (pH 7.5). The reaction was started by adding enzyme solution and the remaining activity was monitored at 25°C.

Temperature Stability. The thermostability of the enzyme was evaluated after preincubation of the enzyme in the same buffer for 50 min at various temperatures (20 to 70°C). Concentrated enzyme solution (10 units) was mixed with 980 μL of the 50 mM Tris buffer (pH 7.5) pre-equilibrated to the desired temperature. An aliquot was taken out at various time intervals and assayed for its remaining activity at 25°C.

Steady-State Kinetics. The substrate specificity was determined for *p*-cresol, L-tyrosine, caffeic acid, (+)-catechin, catechol, L-DOPA (L-3(3,4-dihydroxyphenyl)alanine), DL-dopamine (DL-dihydroxyphenethylamine), 4-methycatechol, protocatechuic acid, gallic acid, and pyrogallol. Reaction mixture (1 mL) contained 50 mM Tris buffer (pH 7.5) and 50 μM substrate. The wavelength for the maximum absorbance of each substrate was obtained by scanning the range of 220–800 nm. Kinetic parameters were determined from the double reciprocal plot of Lineweaver and Burk (1934).

Data Processing. Data for initial velocity studies were fitted to Eq. 1 (Cleland, 1979).

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

Here, v is observed velocity, V_{max} is maximum velocity, K_m is Michaelis-Menten constant and A is substrate concentration.

Results and Discussion

Growth of *Pseudomonas pseudoalcaligenes* KF707. The growth curve of *Pseudomonas pseudoalcaligenes* KF707 shows ca. 4 h of lag phase and ca. 5 h of logarithmic (log) phase as shown in Fig. 1. The maximum specific activity of the protocatechuate 3,4-dioxygenase appeared at the late log phase.

Extraction and Purification of Protocatechuate 3,4-Dioxygenase. The results for fractionation with ammonium sulfate solution are shown in Table 1. 40–50% range of ammonium sulfate shows the maximum protein precipitation. We used 20–80% range of ammonium sulfate for all the extraction processes. Desalted enzyme obtained from dialysis in 50 mM Tris-Acetate buffer (pH 7.5) was injected into a Bio-Scale Q2 column incorporated in a Bio-Rad Automated Protein Purification system. The elution profile is shown in Fig. 2 and shows that protocatechuate 3,4-dioxygenase protein was eluted at 0.34 M NaCl. It was pooled,

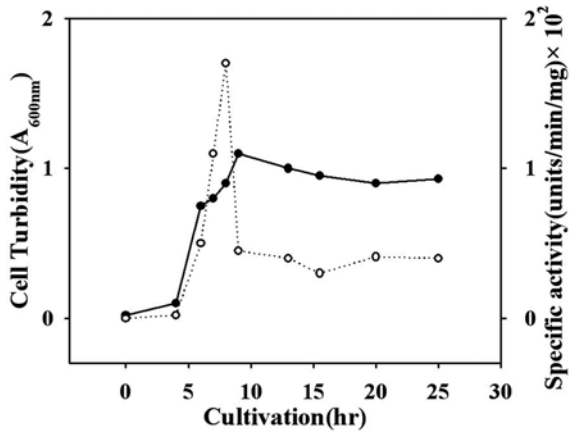


Fig. 1 Growth profile of *Pseudomonas pseudoalcaligenes* KF707. Cell culture was performed as described in Materials and Methods. —, cell turbidity; ••••••, activity.

Table 1 Relative activities of protocatechuate 3,4-dioxygenase from *Pseudomonas pseudoalcaligenes* KF707 by ammonium sulfate fractionation

Ammonium Sulfate Concentration (% Saturation)	Relative Activity (%)
10–20	3.3
20–30	15.4
30–40	18.3
40–50	20.1
50–60	15.4
60–70	14.2
70–80	7.9
80–90	4.2

concentrated and chromatographed in an Econo-Pac Methyl hydrophobic interaction chromatography (HIC) column, which was pre-equilibrated with the same buffer. The enzyme protein was eluted at 0.35 M ammonium sulfate and clearly separated (Fig. 3). The pooled enzyme from the peak was used for all experiments. The results of purification steps are summarized in Table 2. Protocatechuate 3,4-dioxygenase was purified 3.7-fold with a 71.7% yield by ammonium sulfate fractionation, 76.8-fold with 40.7% yield by Bio-Scale Q2, and finally, 296.8-fold with a 33.7% yield by Econo-Pac Methyl HIC chromatography. Purification of 124-fold and 149.1-fold was reported for protocatechuate 3,4-dioxygenase-G and protocatechuate 3,4-dioxygenase-P from *Moraxella* sp., respectively (Sterjiades and Pelmont, 1989). The purified protocatechuate 3,4-dioxygenase from *Pseudomonas pseudoalcaligenes* KF707 had a specific activity of 121.7 U/mg. As shown in Table 3, much lower specific activities have been observed for protocatechuate 3,4-dioxygenases from other bacteria (Fujisawa and Hayaishi, 1968; Hou et al., 1976; Durham et al., 1980; Bull and Ballou, 1981; Chen et al., 1984; Whittaker et al., 1984; Sterjiades and Pelmont, 1989; Hammer et al., 1996; Eulberg et al., 1998) and lower specific activities of 105 U/mg and 106 U/mg have been observed for protocatechuate 3,4-dioxygenases from *Agrobacterium radiobacter* and *Streptomyces* sp. strain 2065, respectively (Hammer et al., 1996: Iwagami et al., 2000).

Molecular Weight. The purified protocatechuate 3,4-dioxygenase from *Pseudomonas pseudoalcaligenes* KF707 on a native polyacrylamide gel electrophoresis (PAGE) gel was obtained as a single band (data not shown). SDS-PAGE gel indicates that two subunit bands with approximate masses of 29.0 and 34.3 kDa were observed, as shown in Fig. 4. The fact that two protein bands are present in equal amounts supports that protocatechuate 3,4-dioxygenase from *Pseudomonas pseudoalcaligenes* KF707 is composed of at least two protomers. The purified protocatechuate 3,4-dioxygenase was chromatographed on a Sephadex G-150 column (15×150 cm) and its retention time was compared with those of molecular-weight standards; the molecular weight of the protein was calculated to be approximately 193 kDa, as shown in Fig. 5. Therefore, the data supports that the enzyme is composed of 3 αβ protomers. This belongs to the lower end of the molecular size range of other protocatechuate 3,4-dioxygenases, 150 kDa to

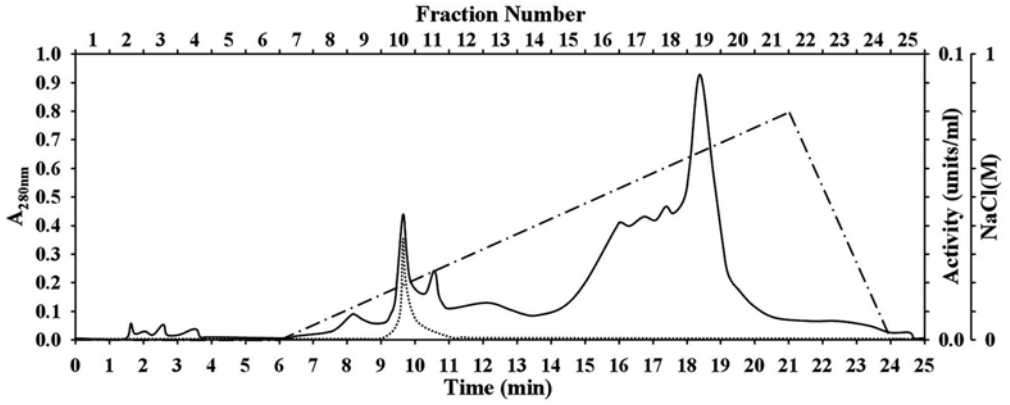


Fig. 2 Elution profile of protocatechuate 3,4-dioxygenase from *Pseudomonas pseudoalcaligenes* KF707 from Bio-Scale Q2 column. Chromatography was performed as described in Materials and Methods. —, protein concentration; ••••••, activity of protocatechuate 3,4-dioxygenase; –•–, NaCl concentration.

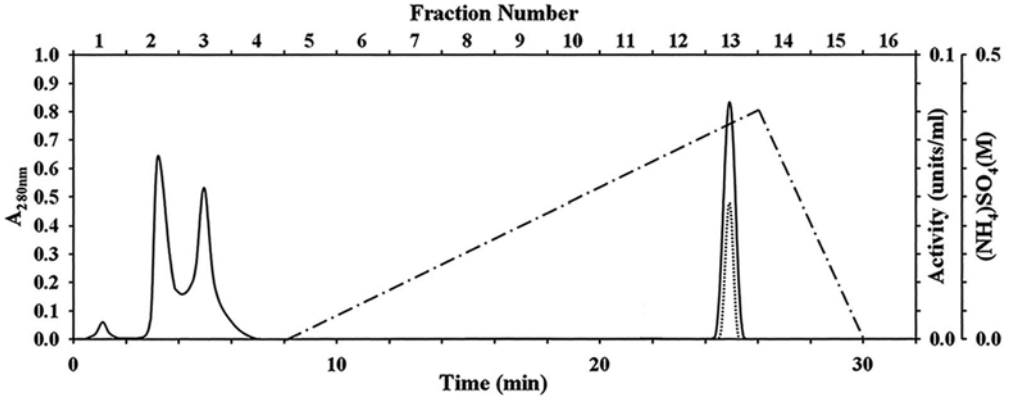


Fig. 3 Elution profile of protocatechuate 3,4-dioxygenase from *Pseudomonas pseudoalcaligenes* KF707 from Econo-Pac Methyl HIC column. Chromatography was performed as described in Materials and Methods. —, protein concentration; ••••••, activity of protocatechuate 3,4-dioxygenase; –•–, $(NH_4)_2SO_4$ concentration.

Table 2 Purification of protocatechuate 3,4-dioxygenase from *Pseudomonas pseudoalcaligenes* KF707

Purification steps	Total Activity (units)	Total Protein (mg)	Specific Activity (units/mg)	Yield (%)	Purification fold
Crude Extract	758.5	1850	0.41	100	1
20–80% $(NH_4)_2SO_4$	543.8	362.5	1.5	71.7	3.7
Bio-Scale Q2[a]	308.7	9.8	31.5	40.7	76.8
Methyl HIC[b]	255.6	2.1	121.7	33.7	296.8

[a]anion exchange column
[b]hydrophobic interaction chromatography

Table 3 Specific activities of protocatechuate 3,4-dioxygenase from various sources

Sources	Specific Activity (U/mg)
Pseudomonas pseudoalcaligenes KF707	121.7
Streptomyces sp. strain 2065[1]	106
Agrobacterium radiobacter[2]	105
Rhodococcus opacus 1CP[3]	53
Azotobacter vinelandii[4]	46.7
Brevibacterium fuscum[5]	41
Pseudomonas putida[6]	33
Acinetobacter calcoaceticus[7]	20
Rhizobium trifolii TA1[8]	0.23
Rhizobium leguminosarum MNF 3841[8]	0.19

[1)]Iwagami et al., 2000; [2)]Hammer et al. 1996; [3)]Eulberg et al., 1998; [4)]Durham et al., 1980; [5)]Whittaker et al., 1984; [6)]Bull and Ballou, 1981; [7)]Hou et al., 1976; [8)]Chen et al., 1984

700 kDa (Fujisawa and Hayaishi, 1968; Hou et al., 1976; Durham et al., 1980; Bull and Ballou, 1981; Chen et al., 1984; Kurane et al., 1984; Whittaker et al., 1984; Sterjiades and Pelmont, 1989; Hammer et al., 1996; Eulberg et al., 1998; Iwagami et al., 2000). The results suggest that the enzyme contains 3 αβ protomers. Protocatechuate 3,4-dioxygenase has been purified from a number of organisms and was always composed of protomers with an equal number of α and β subunits. Each αβ protomer contained one Fe^{3+}. It has been reported that the number of protomers ranged from $(\alpha\beta Fe^{3+})_2$ for *Agrobacterium radiobacter* (Hammer et al., 1996) to $(\alpha\beta Fe^{3+})_{12}$ for *Pseudomonas putida* (Lipscomb and Orville, 1992). The number of αβ protomers per molecule was estimated to be 3 in *Streptomyces sp*. strain 2065 (Iwagami et al., 2000) and 10 in *Azotobacter vinelandii* (Durham et al., 1980). Recombinant protocatechuate 3,4-dioxygenase from *Acinetobacter strain* ADP1 revealed that its stoichiometry is also $(\alpha\beta Fe^{3+})_{12}$ (Vetting et al., 2000).

Effect of pH on the Activity. Protocatechuate 3,4-dioxygenase from *Pseudomonas pseudoalcaligenes* KF707 has activity over the pH range of 4.7–10.5 and the optimum pH value of this enzyme for protocatechuate is pH 7.5 (Fig. 6). The optimum pH values of protocatechuate 3,4-dioxygenases from different sources are as follows: *Pseudomonas putida* (classified as *Pseudomonas aeruginosa* at that time), pH 8.0 (Fujisawa and Hayaishi, 1968); *Chaetomium piluluferum*, pH 7.0 (Wojtas-Wasilewska and Trojanowski, 1980); *Acinetobacter calcoaceticus*, pH 8.5–9.0 (Vetting, 1994). On the other hand, Iwakami et al. (2000) reported that the relative activity of protocatechuate 3,4-dioxygenases from *Streptomyces sp*. strain 2065 increased more than 4.5-fold as the pH increased from 6.5 to 9.5 at increments of 0.5, and that maximum relative activity was detected at pH 9.5. They noted that the optimum pH may be higher, because protocatechuic acid above pH 9.0 undergoes non-enzymatic oxidation (Stanier and Ingraham, 1954). Moreover, the enzyme behaves similarly to enzymes from *Burkholderia cepacia* (Bull and Ballou, 1981) and *Rhizobium trifolii* (Chen et al., 1984), and has a higher optimum pH than enzymes from *Azotobacter vinelandii* (Durham et al.,

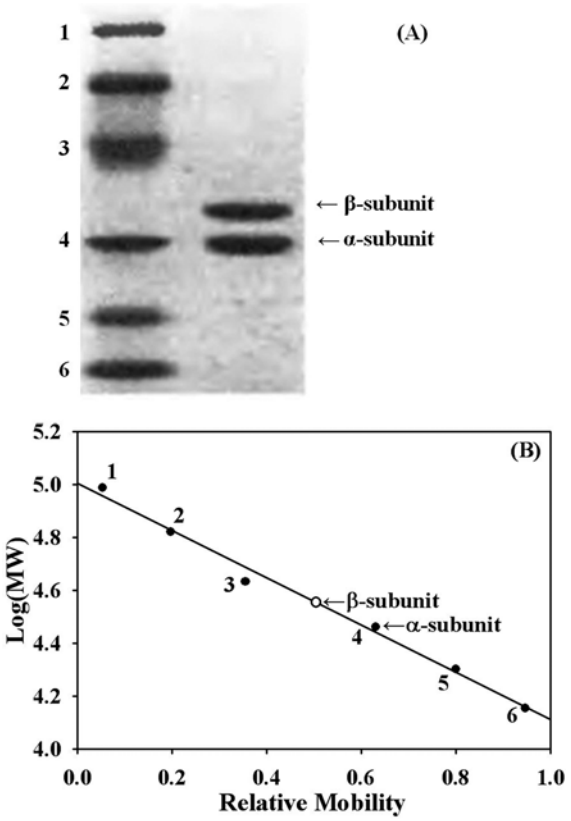


Fig. 4 SDS-PAGE and subnit molecular weight determination of protocatechuate 3,4-dioxygenase from *Pseudomonas pseudoalcaligenes* KF707. SDS-PAGE was performed as described in Materials and Methods. (A) A, rabbit phosphorylase b (MW 97.5 kDa); B, bovine serum albumin (MW 66.2 kDa); C, ovalbumin (MW 43 kDa); D, carbonic anhydrase (MW 29 kDa); E, trypsin inhibitor (MW 20.1 kDa); F, lysozyme (MW 14.3 kDa). (B)The molecular sizes of the a and b subunits of the protocatechuate 3,4-dioxygenase are 29 and 34.3 kDa, respectively.

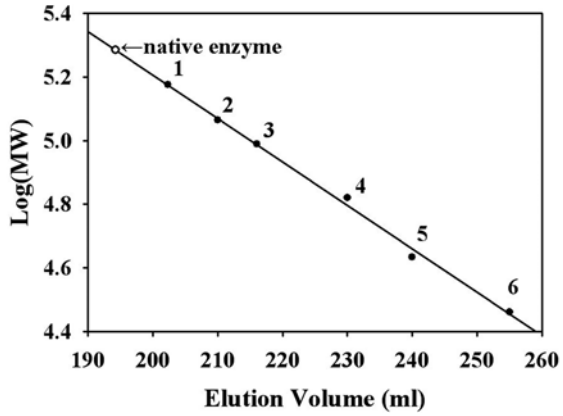


Fig. 5 Native molecular weight determination of protocatechuate 3,4-dioxygenase by Sephadex G-150 column (1.5×100 cm) chromatography. Gel filtration was performed as described in Materials and Methods. A, immunoglobulin G (MW 150 kDa); B, β-galactosidase (MW 116 kDa); C, rabbit phosphorylase b (MW 97.5 kDa); D, bovine serum albumin (MW 66.2 kDa); E, ovalbumin (MW 43 kDa); F, carbonic anhydrase (MW 29 kDa). The native molecular weight of the protocatechuate 3,4-dioxygenase is 193 kDa.

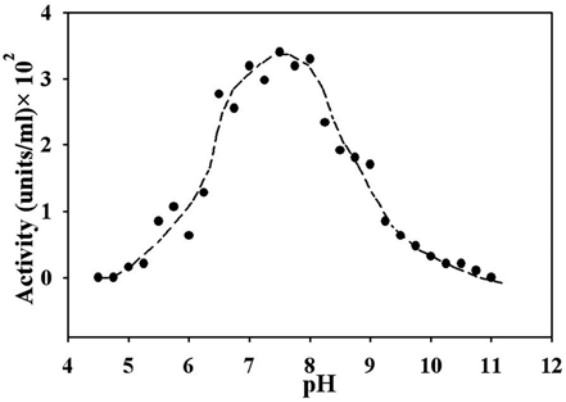


Fig. 6 Effect of pH on the activity of protocatechuate 3,4-dioxygenase from *Pseudomonas pseudoalcaligenes* KF707. Experiment was performed as described in Materials and Methods.

1980) or *Pseudomonas putida* (Fujisawa and Hayaishi, 1968).

Effect of Temperature on the Activity and Stability. The optimum temperature of protocatechuate 3,4-dioxygenase for protocatechuate was determined to be 38°C (Fig. 7A). The enzyme was relatively stable over 20–50°C for 50 min, (Fig. 7B). However, at 70°C, 95% of the activity was lost within 20 min. In the case of protocatechuate 3,4-dioxygenase from *Azotobacter vinelandii*, 90% of the enzymatic activity was lost after 10 min incubation at 60°C (Durham et al., 1980).

Substrate Specificity. The substrate specificity for browning precursors was investigated at each specific wavelength, and the results are shown in Table 4. Purified protocatechuate 3,4-dioxygenase exhibited Michaelis-Menten kinetics for all the substrates tested in this study. K_m values of catechol, protocatechuate, gallate, *p*-cresol, caffeic acid, catechin, L-DOPA, 4-methylcatechol and pyrogallol were 14, 17, 2, 10, 12, 20, 30, 21 and 3 μM, and the V_{max}/K_m (mim^{-1}) values were 0.052, 3.06, 0.35, 0.01, 0.03, 0.02, 0.006, 0.008 and 0.11,respectively. A double reciprocal Lineweaver-Burk plot for protocatechuate as a main substrate is shown in Fig. 8. The K_m value for protocatechuate is similar to that from other sources: 18 μM for *Azotobacter vinelandii* (Durham et al., 1980), 11 μM for *Chartomium piluliferum* (Wojtas-Wasilewska and Trojanowski, 1980) and 30 μM for *Pseudomonas aeruginosa* (Fujisawa and Hayaishi, 1968). However, it is much less than the K_m of 125 μM for *Brevibacterium fuscum* (Whittaker et al., 1984). Compared with the data from *Pseudomonas aeruginosa* (Fujisawa and Hayaishi, 1968), *Acinetobacter calcocaeticus* (Houet al., 1976), *Azotobacter vinelandii* (Durham et al., 1980) and *Chartomium piluliferum* (Wojtas-Wasilewska and Trojanowski, 1980), which did not exhibit any activity on *p*-cresol, L-tyrosine, caffeic acid, (+)-catechin, L-DOPA, DL-dopamine, 4-methylcatechol

Table 4 Substrate specificity of protocatechuate 3,4-dioxygenase from *Pseudomonas pseudoalcaligenes* KF707

Substrate	λ_{max}[a] (nm)	K_m (mM×10^2)	V_{max} (mM/min×10^4)	V_{max}/K_m (mim^{-1}×10^2)
Monophenols				
p-cresol	276	1.0	1.0	1.0
L-tyrosine	275			
Diphenols				
catteic acid	265	1.2	3.6	3.0
(+)-catechin	279	2.0	4.0	2.0
catechol	275	1.4	7.28	5.2
L-DOPA[b]	287	3.0	1.8	0.6
4-methylcatechol	280	2.1	1.68	0.8
protocatechuic acid	258	1.7	520	306
Trihydroxyphenols				
gallic acid	259	0.2	7.0	35
pyrogallol	319	0.3	3.3	11

Activity of protocatechuate 3,4-dioxygenase was measured as described in Materials and Methods.
[a]maximun wavelength, [b]L-3-(3,4-dihydroxyphenyl)alanine.

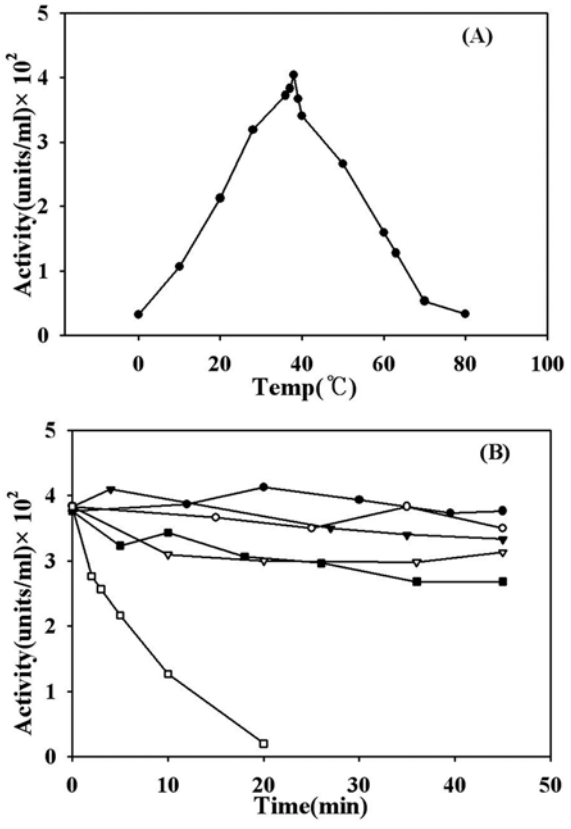


Fig. 7 Effect of temperature on the activity and stability of protocatechuate 3,4-dioxygenase from *Pseudomonas pseudoalcaligenes* KF707. Effect of temperature on the activity. Experiment was performed as described in Materials and Methods. (B) Effect of temperature on the stability. Experiment was performed as described in Materials and Methods. —●—, 20°C; —■—, 30°C; —◆—, 40°C; —◇—, 50°C; —▲—, 60°C; —△—, 70°C.

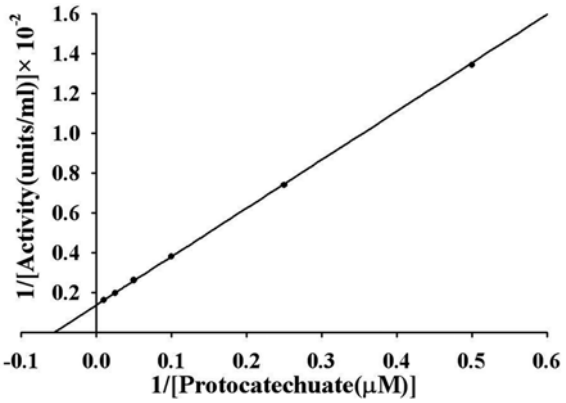


Fig. 8 Double reciprocal plot for protocatechuate 3,4-dioxygenase as a function of protocatechuate concentration. Experiment was performed as described in Materials and Methods.

and pyrogallol, the substrate specificity of protocatechuate 3,4-dioxygenase from *Pseudomonas pseudoalcaligenes* KF707 is relatively much low. Even for catechol as a substrate, protocatechuate 3,4-dioxygenase from *Azotobacter vinelandii* (Durham et al., 1980) and *Moraxella sp*. GU2 (Sterjiades and Pelmont, 1989) showed only 3% and 1.8% activity compared to that of protocatechuate, respectively. Our data show that the turnover number of protocatechuate 3,4-dioxygenase from *Pseudomonas pseudoalcaligenes* KF707 is 68 s^{-1}; much higher than the known values of 41.6 s^{-1} and 6.9 s^{-1} of protocatechuate 3,4-dioxygenase from *Brevibacterium fuscum* (Whittaker et al., 1984) and *Pseudomonas putida* (Bull and Ballou, 1981), respectively. Therefore, our results suggest that protocatechuate 3,4-dioxygenase from *Pseudomonas pseudoalcaligenes* KF707 can be counted as a new anti-browning enzyme. Based on the V_{max}/K_m values, first

order rate constant, protocatechuate was the most efficient substrate of this enzyme. In the following papers, we present data which suggests that the enzyme utilizes a chemical mechanism requiring 5 functional groups (cysteine, carboxylate, histidine, serine, and tyrosine) for the binding of substrates and/or catalysis.

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References

Bedard DL, Unterman R, Bopp LH, Brennan MH, Haberl ML, and Johnson C (1986) Rapid assay for screening and characterizing microorganisms for theability to degrade polychlorinated biphenyls. *Appl Environ Microbiol* **51**, 761–8.

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–54.

Broderik JB and O'Halloran TV (1991) Overproduction, purification, and characterization of chlorocatechol dioxygenase, a nonheme iron dioxygenase with broad substrate tolerance. *Biochemistry* **30**, 7349–58.

Bull C and Ballou DP (1981) Purification and properties of protocatechuate 3,4-dioxygenase From *Pseudomonas putida*. A new iron to subunits toichiometry. *J BiolChem* **256**, 12673–80.

Cleland WW (1979) Statistical analysis of kinetic data. *Methods Enzymol* **63**, 103–8.

Chen YP, Glenn AR, and Dilworth MJ (1984) Uptake and oxidation of aromatic substrates by *Rhizobium leguminosarum* MNF 3841 and *Rhizobium trifolii* TA1. *FEMS MicrobiolLett* **21**, 201–5.

Contzen M, Moore ERB, Blümel S, Stolz A, and Kämpfer P (2000) *Hydrogenophaga Intermedia*sp. nov., a 4-aminobenzenesulfonate degrading organism. *Syst Appl Microbiol* **23**, 487–93.

Davis BJ (1964) Disk electrophoresis. II. Method and applicationto human serum proteins. *Ann NY AcadSci* **121**, 404–27.

Durham DR, Stirling LA, Ornston LN, and Perry JJ (1980) Intergeneric evolutionary homology revealed by the study of protocatechuate 3,4-dioxygenase from *Azotobacter vinelandii*. *Biochemistry* **19**, 149–55.

Eulberg D, Lakner S, Golovleva LA, and Schlömann M (1998) Characterization of a protocatechuate catabolic gene cluster from *Rhodococcusopacus* 1CP: evidence for a merged enzyme with 4-carboxymuconolactone decarboxylating and 3-oxoadipate enol-lactone hydrolyzing activity. *J Bacteriol* **180**, 1072–81.

Finkle BJ and Nelson RF (1963) Enzyme reactions with phenolic compounds: A *meta* methyltransferase in plants. *Biochim Biophys Acta* **78**, 747–54.

Finkle BJ (1964) Treatment of plant tissue to prevent browning.*US Patent* **3**,126,287.

Fujisawa H and Hayaishi O (1968) Protocatechuate 3,4-dioxygenas. I. Crystallizatin and characterization. *J Biol Chem* **243**, 2673–81.

Fujisawa H, Hiromi K, Uyeda M, Okuno S, Nozaki M, and Hayaishi O (1972) Protocatechuate 3,4dioxygenase III. An oxygenated form of the enzyme as reaction intermediate. *J Biol Chem* **247**, 4422–8.

Hammer A, Stolz A, and Knackmuss H (1996) Purification and characterization of a novel type of protocatechuate 3,4-dioxygenase with the ability to oxidize 4-sulfocatechol. *Arch Microbiol* **166**, 92–100.

Hou CT, Lillard MO, and Schwartz RD (1976) Protocatechuate 3,4-dioxygenase from *Acinetobacter calcoaceticus*. *Biochemistry* **15**, 582–8.

Iwagami SG, Yang K, and Davies J (2000) Characterization of the protocatechuic acid catabolic gene cluster from *Streptomyces sp*. strain 2065. *Appl Environ Microbiol* **66**, 1499–508.

Kelly SH and Finkle BJ (1969) Action of a ring-cleaving oxygenase in preventing oxidase darkening of apple juice. *J Sci Food Agr* **20**, 629–32.

Kurane R, Ara K, Nakamura I, Suzuki T, and Fukuoka S (1984) Protocatechuate 3,4 dioxygenase from *Nocardia erythropolis*. *Agric Biol Chem* **48**, 2105–11.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (London)* **227**, 680–1.

Lineweaver H and Burk D (1934) The determination of enzyme dissociation constant. *J Am Chem Soc* **56**, 658–61.

Lipscomb JD and Orville AM (1992) In *Metal Ions in Biological Systems*, Sigel H and Sigel A (Eds.), CRC Press, USA.

Ludwig ML (1984) Characterization of crystals of protocatechuate 3,4-dioxygenase from *Pseudomonas cepacia*. *J Biol Chem* **259**, 14840–2.

Vetting MW, D'argenio DA, Ornston LN, and Ohlendorf DH (2000) Structure of *Acinetobacter* strain ADP1 protocatechuate 3,4-dioxygenase at 2.2 Å. *Biochemistry* **39**, 7943–55.

Mccord JD and Kilara A (1983) Control of enzymatic browning in processed mushrooms (*Agaricusbisporus*). *J Food Sci* **48**, 1479–86.

Ponting JD, Jackson R, and Watters G (1971) Refrigerated apple slices. Effects of pH, sulfites and calcium on texture. *J Food Sci* **36**, 349–50.

Que L Jr (1983) The catechol dioxygenases. *Adv Inorg Biochem* **5**, 167–99.

Peterson E (1996) Molecular cloning and homology modeling of protocatechuate 3,4 dioxygenase from *Pseudomonas marginata*. *Microbiol Res* **151**, 359–70.

Pujar BG (1983) Purification of protocatechuate 3,4-dioxygenase from *Pseudomonas fluorescens* PHK by affinity chromatography. *Indian J Biochem Biophys* **20**, 112–4.

Seib PA (1985) Oxidation, monosubstitution and industrial synthesis of ascorbic acid: A review. *Int J Vit Nutr Res Suppl* **27**, 259–306.

Stanier RY and Ingraham JL (1954) Protocatechuic acid oxidase. *J Biol Chem* **210**, 799–808.

Stanier RY, Palleroni NJ, and Doudoroff M (1966) The aerobic pseudomonads: a taxonomic study. *J Gen Microbiol* **43**, 159–271.

Sterjiades R and Pelmont J (1989) Occurrence of two different forms of protocatechuate 3,4 dioxygenase in a *Moraxella* sp. *Appl Environ Microbiol* **55**, 340–7.

Taira K, Horose J, Hayashi S, and Furukawa K (1992) Analysis of *bph* operon from polychlorinated biphenyl-degrading strain of *Pseudomonas pseudoalcaligenes* KF707. *J Biol Chem* **267**, 4844–53.

Vamos-Vigyazo L (1981) Polyphenol oxidase and peroxidase in fruits and vegetables. *CRC Crit Rev in Food SciNutr* **15**, 158–60.

Vetting MW (1994) Crystallization and preliminary X-ray analysis of protocatechuate 3,4 dioxygenase from *Acinetobacter calcoaceticus*. *J Mol Biol* **236**, 372–3.

Whittaker JW, Lipscomb JD, Kent TA, and Münck E(1984) *Brevibacterium fuscum* protocatechuate 3,4-dioxygenase. Purification, crystallization, and characterization. *J Biol Chem* **259**, 4466–75.

Wojtas-Wasilewska M and Trojanowski J (1980) Purification and properties of protocatechuate 3,4-dioxygenase from *Chaetomium piluliferum* induced with *p*-hydroxybenzoic acid. *Acta Biochim Pol* **27**, 21–34.