Protocatechuate 3, 4-Dioxygenase from *Pseudomonas sp.* : Partial Purification and Some Properties

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Protocatechuate 3, 4-dioxygenase (EC 1. 13. 11. 3) catabolizes the intradiol addition of dioxygen to protocatechuate (3, 4-dihydroxybenzoate), cleaving the aromatic ring to form β-carboxy, cis, cis-muconate. This enzyme (PCD) is an important component of the bacterial metabolism of aromatic compounds. Since Stanier and Ingraham[1] first studied in 1954, PCD has been isolated from several sources including *Pseudomonas*[2,3,4], *Acinetobacter*[5], *Azotobacter*[6], *Thiobacillus*[7], *Nocardia*[8], *and Rhizobium*[9]. Besides the above, it is supposed that *Corynebacterium*[10] includes PCD. Thus PCD is widespread in soil bacteria and each molecular weight is different. These facts suppose that protocatechuate 3, 4-dioxygenase serves the evolutionary study.

We reported that eugenol was metabolized by a *Ps. sp*[11] through intermediates such as coniferylalcohol, ferulate, vanillate and protocatechuate. Extract of eugenol-grown cells showed strong activity to protocatechuate, and then PCD in those cells was purified and tested on some properties comparing with those of the enzymes isolated from other organisms.

Materials and Methods

Microorganisms A eugenol-utilizing bacterium, previously identified as *Pseudomonas sp*[11], was used.

Chemicals DEAE-Sephadex A-25 and Sephadex G-150 were obtained from Pharmacia Fine Chemicals (Sweden). DEAE-Cellulose DE-52 was purchased from Whatman (UK). Other chemicals were reagents of commercial grade.

Cultivation Liquid cultures were grown on a reciprocal shaker at 30°C, in 2-liter shaking flasks containing 600ml of synthetic defined medium[11] with the addition of 0. 5% eugenol (final concentration by feeding) as the sole carbon and energy source.

Preparation of cell-free extract Cells grown on the above medium were har-

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vested and centrifuged continuously at 10,000×g, and then washed three times with 50mM sodiumphosphate buffer (pH 7.2). The cells were suspended in 50mM Tris-HCl buffer (pH 8.0) at a ratio of 20% (w/w). The suspension was sonicated for 6-10 min and centrifuged at 20,000×g for 30min. The supernatant was used as the crude cell-free extract.

Enzyme assay PCD was assayed by oxygen uptake with Warburg's apparatus (Yanagimoto Mfg Co. Ltd., Kyoto, Japan). The reaction mixture was composed of 2.0ml of the enzyme solution in 25mM Tris-HCl buffer (pH 8.0) and 0.5ml (4 μmole) of the substrate solution in the same buffer (final substrate concentration 1.6mM). After incubation for 8-20 min at 30°C, the enzyme activity was estimated from the oxygen consumption measured by a manometer. The endogenous respiration was subtracted.

Purification of PCD Protaminesulphate (*ca* 1.5 mg/ml) was added with mechanical stirring to the crude extract. After stirring for 2 min, the resulting precipitate was removed by centrifugation. The extract was treated with neutral saturated ammonium sulphate, and the material precipitating up to 70% saturation collected by centrifugation after 30 min stirring. The resultant pellet was resuspended in 2ml of 25 mM Tris-HCl buffer (pH 8.0) and dialyzed three times against the same buffer at 5°C using a visking tube (20/30). The dialysate was loaded on a column of DEAE-cellulose DE-52 previously equilibrated with the same buffer. After washing with same buffer (containing 0.05M NaCl), the column was eluted with a linear gradient of NaCl over the range 0.05-1.0 M in the same buffer. The peak fractions of PCD activity were pooled, and was retreated with ammonium sulphate at 70% saturation. After centrifugation, the pellet was suspended in 2 ml of the same buffer and dialyzed against the same buffer using a visking tube.

The dialysate was loaded on a column of DEAE-Sephadex A-25 equilibrated with the 25 mM Tris-HCl buffer (pH 8.0), and eluted in the same manner as in the DEAE-cellulose column chromatography. The active fractions from A-25 column were concentrated with a visking tube. The concentrated sample was loaded on a column of Sephadex G-150 previously equilibrated with the same Tris-HCl buffer (pH 8.0, 0.1M NaCl).

Results

I. *Purification of protocatechuate 3,4-dioxygenase*

The cell-free extract which was described above was used to purify the enzyme. The overall purification process is summarized in Table I. The final enzyme was purified about 122-fold from the original crude extract. When this

Table I Purification of Protocatechuate 3,4-Dioxygenase from *Ps. sp.*

Step	Vol. ml	OD_{280}	Total activity (units)	Specific activity (units/OD_{280}/ml)	Relative activity
Crude extract	50	77.2	4709.2	1.22	1
Protamine sulphate	50	34.8	4506.6	2.59	2.12
Ammonium sulphate	20	44.4	3303.4	3.72	3.05
DEAE Cellulose	170	1.04	2300.2	13.01	10.68
DEAE Sephadex	100	0.28	1400.0	50.00	41.06
Sephadex G-150	7	0.22	230.0	149.35	122.66

1 unit=3 μl O_2 uptake at 35°C/min

material was retreated with Sephadex G-150 (the last step), the purification was raised by more than several thousand-fold.

II. *Effect of nutrient*

Bouillon, peptone and yeast extract were tested for effect of PCD activation. PCD activity was identified in crude extract from bouillon-grown cells

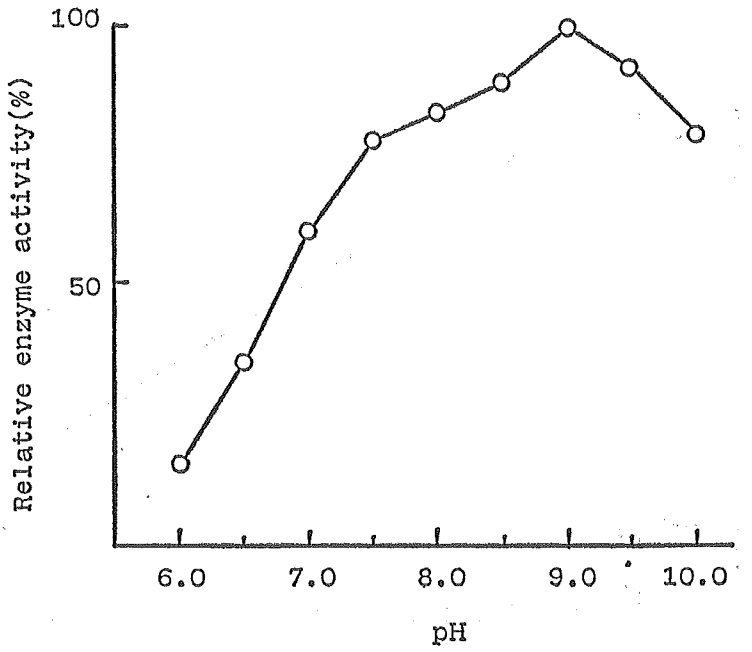

Fig. 1. Effects of pH on the activity of Protocatechuate 3,4-dioxygenase. The following buffers were used for determination in each pH region : pH 6.0-7.5, phosphate ; pH 8.0-9.0, Tris-HCl ; pH 9.5-10.0, carbonate.

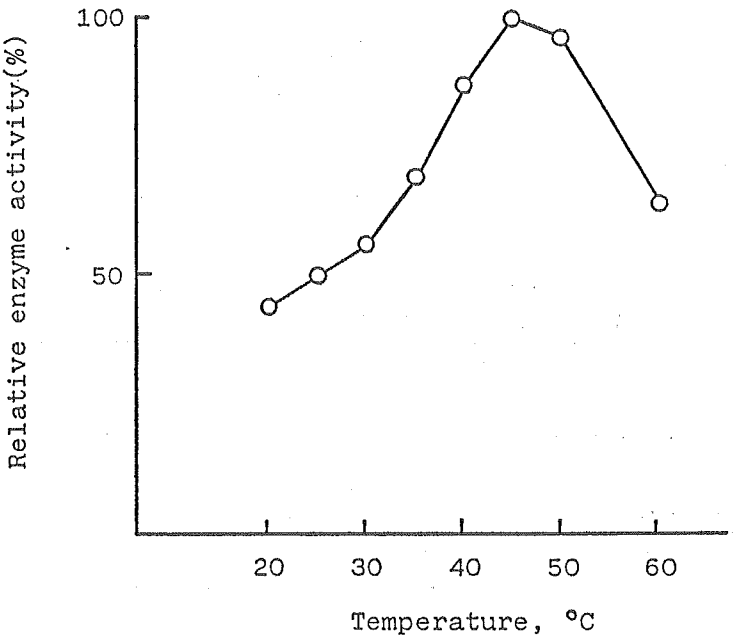

Fig. 2. Effects of temperature on the activity of Protocatechuate 3, 4-dioxygenase.

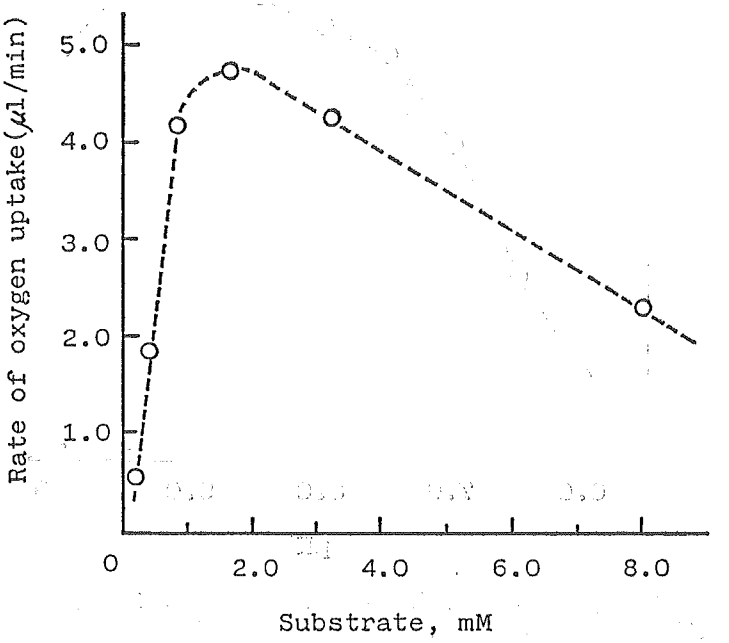

Fig. 3. Plot of oxygen-uptake rate *vs* substrate concentration.

at *ca* 10% of that of the eugenol-grown cells. Eugenol-grown cells, however, were not positively affected by adding 0.01% of nutrients.

III. *Properties of protocatechuate 3,4-dioxygenase*

Effects of pH The enzyme obtained was most active at around pH 9.0, shown in Fig. 1.

Effects of temperature The enzyme was more active at around 45°C, shown in Fig. 2.

Michaelis constant The rate of oxygen uptake was reached to the maximum around 2 mM of protocatechuate concentration, and decreased with increasing its concentration (Fig. 3). The apparent K_m for protocatechuate from this figure was estimated to be *ca* 5×10^{-4} M.

Substrate specificity As shown in Table II. tested materials including o-dihydroxylphenyl compound were not served as the substrate for this enzyme under these conditions.

Table II Substrate Specificity

Substrate (1.6mM)	Relative enzyme activity (%)
Protocatechuate	100
Eugenol	0
Ferulate	0
Vanillate	0
Phthalate	0
Salicylate	0
m-Hydroxy benzoate	0
p-Hydroxy benzoate	0
Resorcin	0
Pyrogaroll	0
Hydroquinone	0
Catechol	0
4-Methyl catechol	0
3-Methyl catechol	0

Discussion

Protocatechuate 3,4-dioxygenases from *Pseudomonas*, *Acinetobacter*, *Azotobacter*, *Thiobacillus* and *Rhizobium* have roughly constant K_m-values, 3.0, 7.1, 1.8, 6.6 and 1.75 ($\times10^{-5}$), respectively. Durham *et al* has described that the above coincidence and resemblance of amino acid composition between *Azotobacter* and, *Pseudomonas* and *Thiobacillus* favoured the conclusion that the bacterial protocatechuate 3,4-dioxygenase were derived from a common ancestral

protein. The enzyme which we obtained, however, had larger K_m-value than others. Though the molecular weight of protocatechuate 3,4-dioxygenase was not estimated precisely (larger than several hundreds thousand), a new data of K_m-value might bring about novel informations. With substrate specificity for the obtained enzyme, the degree is on a higher level. On this point, our enzyme has especial characteristics, and may have a new potentiality for further investigation.

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Pseudomonas sp. からのプロトカテキン酸 3,4-二原子酸素添加酵素：部分精製および諸性質について

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プロトカテキン酸　3, 4-二原子酸素添加酵素に関しては，芳香族化合物の微生物的代謝や微生物進化などの点から多くの報告が見られ，その菌種も多岐にわたっている。土壌から分離したオイゲノール資化性菌である *Pseudomonas* 属細菌にもこの酵素が認められた。この酵素は基質であるプロトカテキン酸の高濃度範囲では活性が一部阻害され，見かけの K_m 値は 5×10^{-4} と，他菌種由来のそれよりも大きい。さらにこの酵素は，かなり広範囲の基質類似化合物に対して活性を示さず，特異性が高いことがわかった。これらの観点から，この酵素は従来のものとは相違すると考えられ，さらに新しい知見が得られることが期待される。