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Short Communication

Catechol 1,2-dioxygenase from *Aspergillus niger*: Purification and properties

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Abstract

Catechol 1,2-dioxygenase was partially purified to 60-fold from cell-free extracts of *Aspergillus niger* grown on anthranilic acid. The purified enzyme was found to be highly unstable. The effects of pH, temperature, metal ions and metal chelating agents on enzyme activity have been studied. The enzyme catalyzes the oxidation of catechol and 4-methyl catechol at the same rate, but does not catalyze the oxidation of 3-methyl catechol, 3-isopropyl catechol and other substrate analogues. The enzyme is completely inhibited by thiol compounds and not affected by thiol inhibitors. The km value for catechol was found to be $5{\cdot}2 \times 10^{5}$ M.

Key words : Catechol 1,2-dioxygenase, *Aspergillus niger*..

1. Introduction

Catechol 1,2-dioxygenase (E.C. 1.13.1.1), catalyzes the intradiol cleavage of the aromatic ring of catechol to yield *cis, cis*-muconic acid with the incorporation of both the atoms of moleeular oxygen into the substrate. It represents the initial enzyme of *β*-ketoadipate pathway, a metabolic sequence used by microorganisms for the degradation of aromatic compounds[1]. The enzymes of the *β*-ketoadipate pathway, including catechol 1,2-dioxygenase, are inducible in microorganisms. Bacteria and fungi utilize *β*-ketoadipate pathway for the transformation of catechol, protocatechuate, and their respective precursors to succinate and acetyl-CoA.

Catechol 1,2-dioxygenase has been purified from *Pseudomonas species*[2], *Brevibacterium fuscum*[3] and *Acinetobacter calcoaceticus*[4]. However, the purification and properties of this enzyme from *Aspergillus niger* (fungi) have not been reported so far. The present work reports it.

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2. Materials and methods

2.1. *Organism*

A. niger strain UBC 814 was the organism used. It was grown on a chemically defined medium supplemented with 0·1% anthranilic acid. Details of the medium and the cultivation of the organism are described in ref. 5.

2.2. *Enzyme assay*

Enzyme activity was determined colorimetrically by measuring the amount of catechol disappearance as described by Nair and Vaidyanathan[6]. It was also determined spectrophotometrically, by measuring the increase in absorbance at 260 nm due to the formation of product *cis, cis*-muconic acid or polarographically, by measuring oxygen uptake with an oxygen electrode.

One unit of enzyme activity is defined as the amount that catalyzes the transformation of 1 μ mole of substrate per min under standard assay conditions (1 Unit = 1000 milliunits). Specific activity is expressed as units per milligram of protein. The protein concentration was estimated by the method of Lowry *et al*[7].

Table I

Purification of catechol 1,2-dioxygenase

Step	Volume (ml)	Activity (mμ)	Protein (mg)	Specific activity (mμ/mg)	Fold purification	Yield %
1. Crude extract	200	13,800	26,500	0·52	..	100
2. Protamine sulphate treatment	200	12,600	10,500	1·2	2·3	91
3. 40–80% NH_4SO_4 fractionation	25	10,825	5,154	2·1	4·0	78
4. DEAE-cellulose chromatography	40	4,645	625	13·83	26·6	63
5. Sephadex G-150 chromatography	26	2,356	72·6	32·45	62·4	17

2.3. *Purification of catechol* 1,2-*dioxygenase*

Purification was carried out in 0·025 M Tris HCl buffer, pH 8·2, at about 4° C. Freshly harvested mycelia (60 g) were ground with an equal amount of glass powder for 15 min and extracted with 180 ml of buffer. The extract was passed through cheese cloth and centrifuged at 12,000 × g for 20 min. Protamine sulphate (20 ml of 2% aqueous solution) was added, with gentle stirring, to 200 ml of crude extract. After standing for 15 min, the precipitate was discarded and the supernatant solution was brought to 40% saturation with ammonium sulphate. Precipitated protein was removed by centrifugation, and the supernatant liquid was brought to 80% saturation with ammonium sulphate. The precipitate formed was collected by centrifugation and dissovled in buffer. This preparation was desalted by passing through sephadex G-25 column and then loaded on to the DEAE-cellulose column (2 × 15 cm) that has been equilibrated with 0·025 M Tris HCl buffer pH 8·2 and washed with the same buffer containing NaCl in a linear gradient running from a concentration of 0 to 0·5 M. Fractions of 5 ml were collected at a flow rate of 60 ml/hr and concentrated by lyophilization. The concentrated DEAE-cellulose eluate (5 ml) was loaded on to sephadex G-150 column (1·5 × 30) that had been equilibrated with buffer. The flow rate was maintained at 25 ml/hr and fractions of 3 ml were collected. Fractions containing enzyme activity were pooled and used as an enzyme preparation for further studies.

Polyacrylamide gel electrophoresis of the final purified preparation at pH 8·6 on 7% acrylamide gels by the method of Davis revealed the existence of at least three proteins (one major and two minor).

Table II

Effect of thiol compounds and thiol inhibitors on catechol 1,2-dioxygenase activity

Substance added	Concentration (mM)	% Inhibition
None		0
GSH	1·0	95
2-Mercaptoethanol	1·0	92
L-cysteine	1·0	90
DTT	1·0	50
PHMB	0·1	10
Iodoacetamide	0·1	0
NEM	0·1	0
Arsenite	0·1	8

3. Results and discussion

Although the crude enzyme was stable in Tris-HCl buffer, pH 8·2 for few weeks at 20° C, the final purified enzyme preparation was highly unstable and loses its activity completely within two days upon storage. In contrast, the purified enzyme from bacteria is stable for considerable periods. The enzyme had a broad pH optimum ranging from pH 7 to 9. The enzyme was unstable at acidic pH. The enzyme activity was found to be optimum at 35° C and decreased rapidly above 45° C.

Among various metal ions tested, only Zn^{2+}, Ag^{+}, and Cu^{2+} inhibited enzyme activity. The enzyme did not show any requirement of metal ions externally for its activity. Its activity was not inhibited by various metal chelating agents such as EDTA, *o*-phenanthroline, diethylthiocarbamate, *a,a*-bipyridyl, salicylaldoxime and Bathocuproin.

At 1 mM concentration, thiol compounds such as reduced glutathione, 2-mercaptoethanol, dithiothreitol and cysteine inhibited the enzyme reaction completely, whereas thiol inhibitors such as PHMB, N-ethylamaleimide, iodoacetate, iodoacetamide and arsenite did not inhibit the reaction. In contrast, the catechol 1,2-dioxygenase from *Acinetobactor calcoaceticus*[4] or *Pseudomonos* species are not inhibited by thiol compounds.

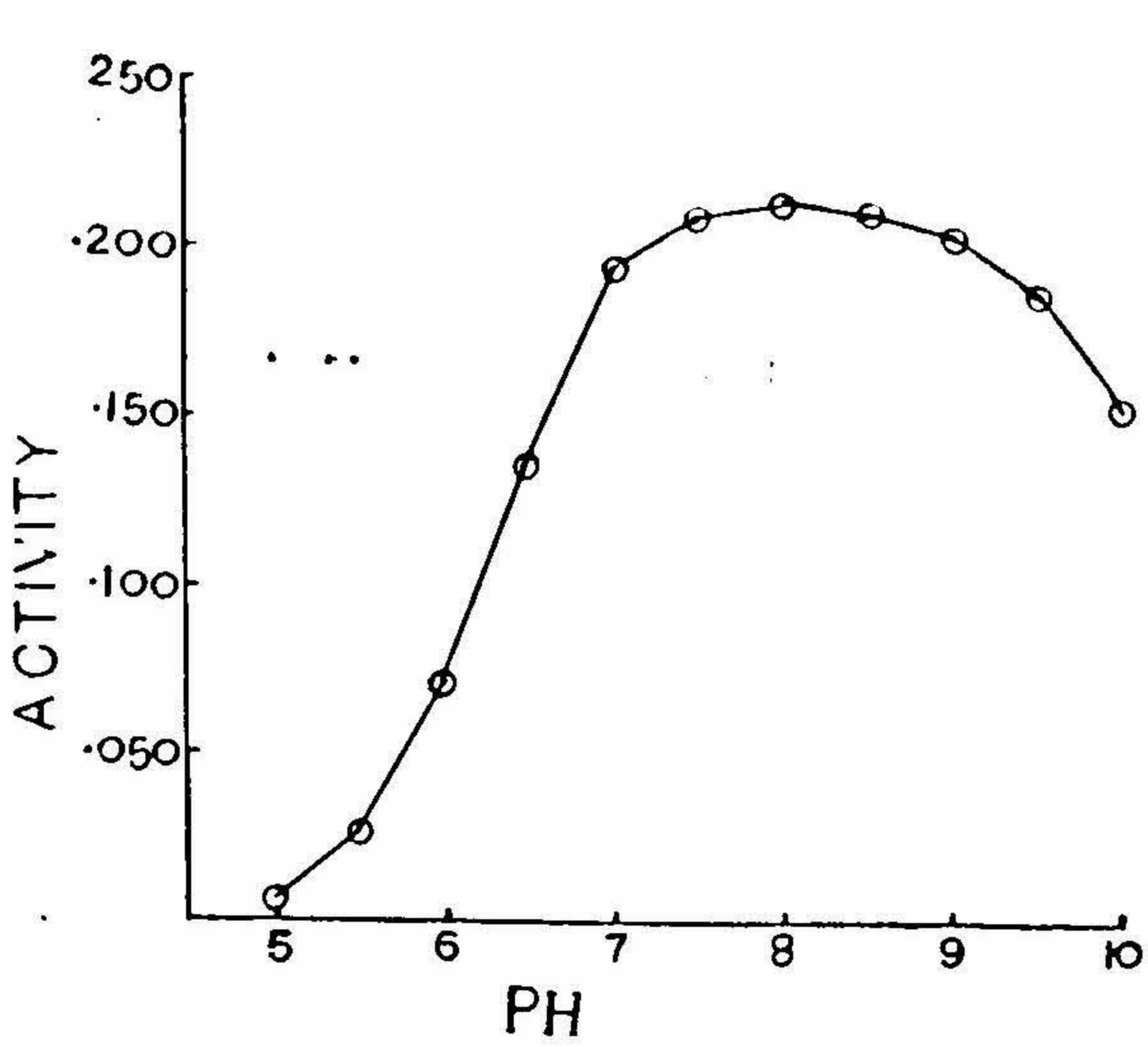

FIG. 1. Effect of pH on catechol 1,2-dioxygenase. Buffers used were potassium phosphate (pH 6·0 to 7·5), Tris HCl (pH 8 to 9) and Glycine : NaOH (pH 9 to 10). Activity was measured as μ moles of catechol disappeared per min.

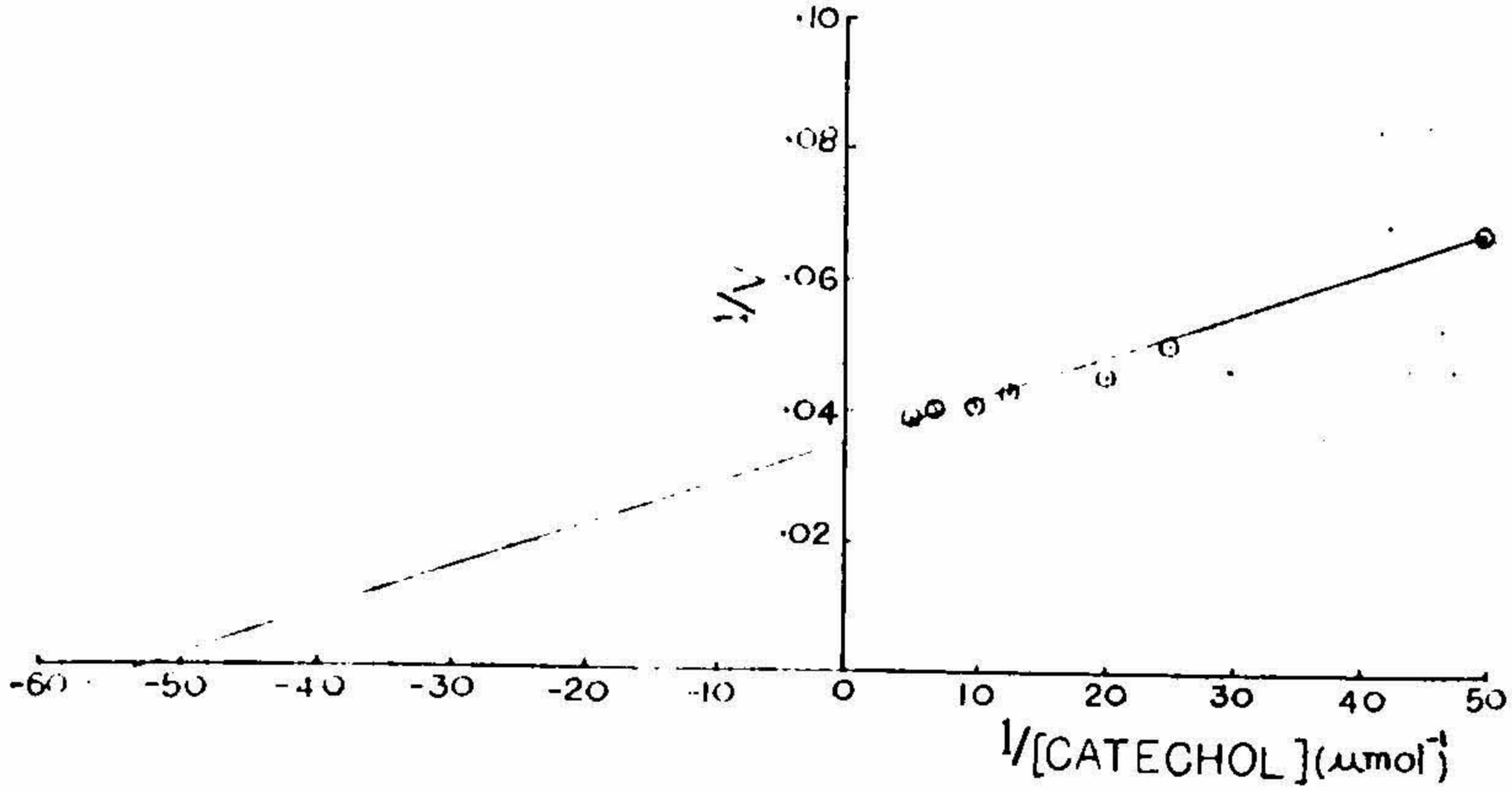

FIG. 2. A Lineweaver-Burk plot. Activity of catechol 1,2-dioxygenase was measured as μ moles of O_2 consumed/min.

The substrate specificity of catechol 1,2-dioxygenase from various microorganisms is characteristically different. The enzyme from *A. niger* (fungi) oxidized catechol and 4-methyl catechol at the same rate and did not oxidize either 3-methyl catechol or isopropyl catechol. In contrast to *A. niger*, the enzyme from *Brevibacterium*[3] oxidized catechol, 4-methyl catechol and 3-methyl catechol at the same rate, whereas the enzyme from *Acinetobacter*[4] catalyzed the oxidation of 4-methyl and 3-methyl catechol at rates 18 and 12% that of catechol, respectively. The enzyme from *Pseudomonas*[2] oxidized catechol and 4-methyl catechol at the same rate and 3-methyl catechol at a rate of 5% that of catechol.

Recently, Fujiwara *et al*[8] reported that the catechol 1,2-dioxygenase from *Pseudomonos* catalyzed the extradiol cleavage of 3-substituted catechols in addition to its known intradiol cleavage activity. In contrast, the enzyme from *Brevibacterium*[9] catalyzed only intradiol cleavage of 3-substituted catechols. However, the catechol 1,2-dioxygenase from *A. niger* did not cleave the 3-substituted catechols.

The catechol 1,2-dioxygenase from *A. niger* showed a linear Lineweaver and Burk plot for catechol with a km value to be $5{\cdot}2 \times 10^{-5}$ M which is higher than that of *Pseudomonas*[2] enzyme ($4{\cdot}0 \times 10^{-6}$).

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