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Purification and Properties of Catechol 1,2-Dioxygenase from *Rhizobium leguminosarum* biovar *viceae* USDA 2370

YUNG PIN CHEN AND CHARLES R. LOVELL*

Department of Biological Sciences, University of South Carolina, Columbia, South Carolina 29208

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The catechol 1,2-dioxygenase of *Rhizobium leguminosarum* biovar *viceae* USDA 2370 was purified 296-fold, yielding a homogeneous preparation with a specific activity of 51.1 U mg of protein^{-1}. The molecular weight of the native protein was 70,000, with two identical subunits of 34,500 and 1 g-atom of iron per mol. The optimum pH for catalytic activity was 9.0 to 9.5.

Catabolism of a variety of aromatic compounds by rhizobia proceeds through initial conversion to catechol or protocatechuate. The aromatic ring of catechol is then opened by catechol 1,2-dioxygenase (catechol:oxygen 1,2-oxidoreductase [decylizing]; EC 1.13.1.1), yielding *cis,cis*-muconate, which is subsequently further catabolized (3, 16). *Rhizobium* strains can differ substantially in the pathways they use to catabolize aromatic compounds (2, 15, 16). The high degree of taxonomic relatedness between *Rhizobium leguminosarum* biovar *trifolii* TA1 (strain TA1) and *R. leguminosarum* biovar *viceae* USDA 2370 (strain 2370) would promote the expectation that their enzymes and pathways of aromatic catabolism would be very similar. These strains have been found to vary significantly in both the properties of their catechol 1,2-dioxygenases and their characteristic catechol and protocatechuate dioxygenases.

Strain 2370, which nodulates *Pisum sativum*, was obtained from the U.S. Department of Agriculture, Beltsville, Md., and maintained on the mineral salts medium of Brown and Dilworth (1). Cultures for catechol 1,2-dioxygenase purifications were grown at 30°C on mineral salts medium with sodium benzoate (3 mM) as the sole carbon source. Catechol 1,2-dioxygenase was assayed by the method of Nakazawa and Nakazawa (12), with catechol as the substrate. Protein was assayed by the method of Lowry et al. (9). Samples of 60 g (wet weight) of benzoate-grown cells in the early stationary phase were used in each purification. Preparation of crude extract, streptomycin sulfate-treated extract, and the 10 to 40% ammonium sulfate fraction was as described by Chen et al. (3). The ammonium sulfate fraction was dialyzed against 50 mM Tris hydrochloride (pH 8.5), insoluble materials were removed by centrifugation, and the supernatant (22 ml) was chromatographed on a 1.6- by 17-cm ion-exchange (DE-52 DEAE-cellulose) column (Whatman LabSales, Hillsboro, Oreg.) that had been preequilibrated with 50 mM Tris hydrochloride (pH 8.6) (CD buffer). Proteins were eluted with a linear gradient (300 ml total) from 0 to 0.4 M NaCl in CD buffer. The enzyme was precipitated from fractions containing peak activity with solid ammonium sulfate; the precipitate was recovered by centrifugation, dissolved in 2 ml of CD buffer, and applied to a 56- by 1.2-cm Sephacryl S300 (Sigma Chemical Co., St. Louis, Mo.) column. CD

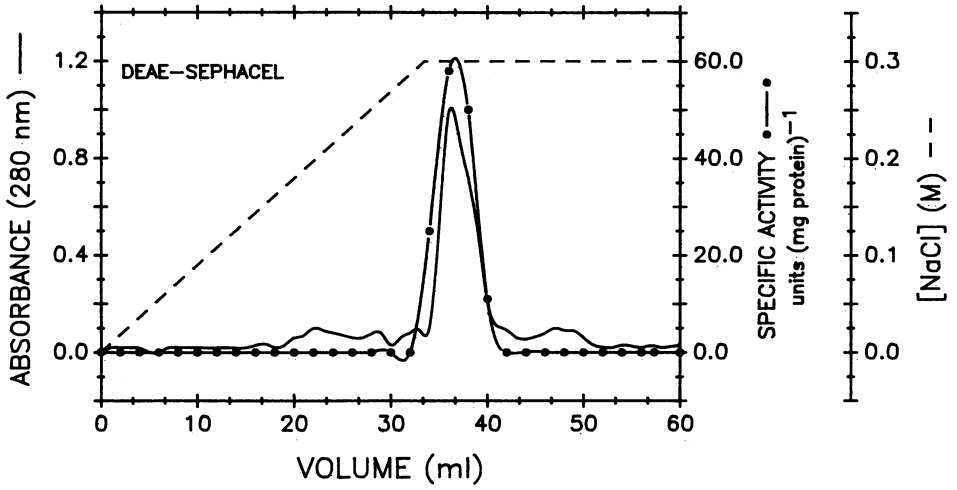

FIG. 1. Purification of *R. leguminosarum* biovar *viceae* USDA 2370 catechol 1,2-dioxygenase on DEAE-Sephacel. Peak fractions from a Sephacryl S300 column were pooled and loaded on the column. The column was eluted with a linear gradient from 0 to 0.3 M NaCl in 50 mM Tris hydrochloride (pH 8.6), followed by 0.3 M NaCl in the same buffer

* Corresponding author.

TABLE 1. Purification of catechol 1,2-dioxygenase from *R. leguminosarum* biovar *viceae* USDA 2370

Treatment	Vol (ml)	Protein		Activity[a]			Sp act (U mg^{-1})	Purification (fold)
		Concn (mg ml^{-1})	Total (mg)	Concn (U ml^{-1})	Total (U)	Recovery (%)		
Crude extract	138	14.3	1,973	2.6	358.8	100	0.178	1
Streptomycin sulfate (6 mg ml^{-1})	123	13.2	1,624	2.7	332.1	92	0.20	1.2
Ammonium sulfate (10 to 40%)	22	13.0	286	15.0	330.0	91	1.2	6
DEAE-cellulose	21	1.2	25	11.4	239.4	66	9.65	54
Sephacryl S300	8	0.38	3.04	10.0	80.0	22	26.6	149
DEAE-Sephacel	3	0.33	0.99	16.8	50.4	14	51.1	296

[a] One unit of catechol 1,2-dioxygenase activity is defined as 1 μmol of substrate oxidized mg of protein^{-1} min^{-1}.

buffer was used to elute the proteins. Fractions containing peak activity were pooled and chromatographed on a 6- by 1.8-cm DEAE-Sephacel (Sigma) column. Catechol 1,2-dioxygenase was eluted with a linear gradient from 0 to 0.3 M NaCl in CD buffer. Elution profiles of the DEAE-cellulose (data not shown), Sephacryl S300 (data not shown), and DEAE-Sephacel (Fig. 1) columns showed the enzyme activity to be confined to a single peak in each chromatograph. The results of the purification are summarized in Table 1. Proteins in crude extract and pooled fractions from each column were electrophoresed on a sodium dodecyl sulfate-polyacrylamide slab gel (15% polyacrylamide) (Fig. 2). The samples were denatured by boiling in Laemmli sample buffer for 5 min before loading (8), and proteins were visualized with Coomassie brilliant blue. The purified strain 2370 catechol 1,2-dioxygenase had a specific activity of 51.1 U mg^{-1}, the highest reported to date.

Estimates of catechol 1,2-dioxygenase molecular weight were obtained from relative mobility in native 10 and 15% polyacrylamide slab gels (5) and from liquid chromatography on Sephacryl S300 columns. Subunit molecular weight was estimated from relative mobility on denaturing sodium dodecyl sulfate-polyacrylamide gels (8). A molecular weight of 70,000 was estimated for the native enzyme from strain 2370, based on both gel filtration and slab gel electrophoresis. A subunit molecular weight of 34,500 was estimated from sodium dodecyl sulfate-polyacrylamide gel electrophoresis. These data are consistent with two identical subunits with molecular weights of about 35,000 forming the holoenzyme. The strain 2370 catechol 1,2-dioxygenase is thus significantly smaller than the strain TA1 enzyme, which had a native molecular weight of 107,000, with two identical subunits with molecular weights of 59,000 each (3).

The iron content of the enzyme was determined by atomic absorption spectrometry (model 965 Plasma Atomcomp; Jarrell-Ash Co., Waltham, Mass). Given a native molecular weight of 70,000, 0.9 g-atom of iron per mol of enzyme was found. Assuming some loss of iron during purification of the enzyme, the true iron content is 1 g-atom of iron per mol, corresponding to that of the similarly sized *Brevibacterium fuscum* (10), *Pseudomonas arvilla* (7), and *Pseudomonas putida* (11) enzymes but half that of the larger strain TA1 protein (3). Amino acid composition was determined from pure strain 2370 enzyme by hydrolysis of 400 pmol of protein in 6 N HCl at 110°C for 24 h under N_2, followed by analysis with a Beckman System 6300 amino acid analyzer (Beckman Instruments, Palo Alto, Calif.). The native protein contains around 652 amino acids, consistent with the empirically determined native protein and subunit molecular weights. The amino acid composition (residues per molecule) is as follows: Ala, 59; Arg, 37; Asx, 76; Glx, 43; Gly, 70; His, 27;

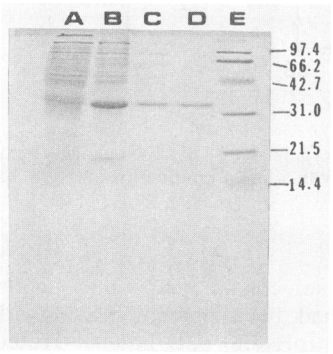

FIG. 2. Protein profiles of the following: A, crude extract; B, pooled DEAE-cellulose column fractions containing peak catechol 1,2-dioxygenase activity; C, pooled Sephacryl S300 fractions containing peak activity; D, pooled DEAE-Sephacel fractions containing peak activity; and E, molecular weight standards fractionated on a sodium dodecyl sulfate-polyacrylamide gel. Molecular weights are indicated in thousands.

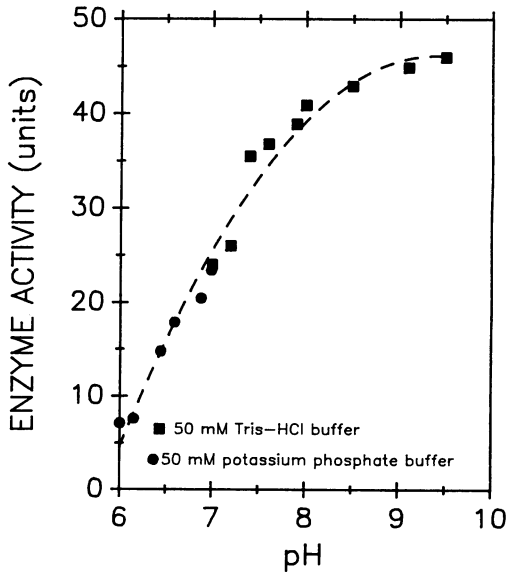

FIG. 3. Activity of catechol 1,2-dioxygenase from *R. leguminosarum* biovar *viceae* USDA 2370 in reaction mixtures buffered at different pHs with either 50 mM Tris hydrochloride or 50 mM potassium phosphate.

TABLE 2. Specific activities of aromatic ring cleavage enzymes[a] in crude extracts of *R. leguminosarum* biovar *viceae* USDA 2370 grown on various aromatic substrates

Substrate	Sp act (U mg of protein^{-1})			
	Cat 1,2-O_2ase	Cat 2,3-O_2ase	Proto 3,4-O_2ase	Proto 4,5-O_2ase
Benzoate	0.178	0	0	0
Catechol	0.184	0	0	0
4-Hydroxybenzoate	0	0	0.47	0.28
Protocatechuate	0	0	0.50	0.30

[a] Cat 1,2-O_2ase, catechol 1,2-dioxygenase; Cat 2,3-O_2ase, catechol 2,3-dioxygenase; Proto 3,4-O_2ase, protocatechuate 3,4-dioxygenase; Proto 4,5-O_2ase, protocatechuate 4,5-dioxygenase.

Ile, 51; Leu, 60; Lys, 23; Met, 6; Phe, 28; Pro, 42; Ser, 34; Thr, 46; Tyr, 14; Val, 36.

The optimum pH for catalysis was determined by assaying pure catechol 1,2-dioxygenase activity in a series of reaction mixes buffered to pH values from 6 to 9.5 with 50 mM Tris hydrochloride or 50 mM potassium phosphate (Fig. 3). Activity increased over six fold from pH 6 to 9.5, with an optimum pH for catalysis of 9.0 to 9.5. This is significantly higher than that of any of the *Pseudomonas* enzymes (6, 7, 11) but very similar to the pH optimum of the strain TA1 enzyme (3). The Strain 2370 enzyme had a K_m for catechol of 20 μM.

Cultures (500 ml) of strain 2370 were grown on the mineral salts medium with benzoate, catechol, 4-hydroxybenzoate, or protocatechuate (3 mM) as the sole carbon source. Crude extracts were assayed for catechol 1,2-dioxygenase (see above), catechol 2,3-dioxygenase (13), protocatechuate 3,4-dioxygenase (4), and protocatechuate 4,5-dioxygenase (14). Specific activities of the dioxygenases found in these crude extracts are given in Table 2. The *ortho*-cleavage enzymes for both catechol (catechol 1,2-dioxygenase) and protocatechuate (protocatechuate 3,4-dioxygenase) were found in extracts of benzoate- or catechol-grown or of 4-hydroxybenzoate- or protocatechuate-grown strain 2370 and in strain TA1 (2), whereas *R. leguminosarum* MNF 3841 (2) and *Rhizobium* sp. (cowpea) strain NGR 234 (15) had only the *ortho*-cleavage enzyme for protocatechuate. No *R. leguminosarum* strains have been reported to carry out the *meta* cleavage of catechol, but the *meta*-cleavage enzyme for protocatechuate (protocatechuate 4,5-dioxygenase) was found in strain 2370 grown on 4-hydroxybenzoate or protocatechuate. Significant variation in the dioxygenases used in aromatic catabolism by closely related rhizobia clearly exists. Future studies of the structure and regulation of expression of the genes encoding rhizobial catechol 1,2-dioxygenases may help establish a basis for this variability.

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