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PHENYLACETIC ACID *META* HYDROXYLASE FROM *RHIZOCTONIA SOLANI**

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Key Word Index—*Rhizoctonia solani*; Agonomycetes; mono-oxygenase; *meta*-position specific hydroxylation of phenylacetic acid; *m*-hydroxyphenylacetic acid.

Abstract—Mono-oxygenase-like activity occurs in *Rhizoctonia solani* during the metabolism of phenylacetic acid. The partially purified enzyme catalyzed only the hydroxylation of phenylacetic acid at its *meta* position and required NADH and tetrahydrofolate as co-factors. Benzoic, phenylpropionic, *trans*-cinnamic, and phenoxyacetic acids were not suitable substrates for the enzyme.

INTRODUCTION

Rhizoctonia solani Kühn, the causal fungus of a serious soilborne disease of plants, is known to produce phenylacetic acid and its hydroxy-derivatives in culture; these metabolites are toxic to plants [1]. Among a number of phytopathogenic fungi so far tested, only *R. solani* has the ability to hydroxylate phenylacetic acid exclusively at the *meta* position, and only phenylacetic acid is a good substrate for the specific hydroxylation [2,3].

In a typical replacement culture with phenylacetic acid, the yield of *m*-hydroxyphenylacetic acid was found to be more than 50% [2]. Although the biosynthesis of *m*-hydroxyphenylacetic acid has been established by radiotracer experiments [3], the enzymatic mechanism has received little attention perhaps because the product is an unusual metabolite. As indicated in recent reviews [4,5], enzymes associated with hydroxylation of aromatic compounds have rarely been detected in fungi, and have not been isolated or characterized. The aim of the present work was to examine the *meta*-specific hydroxylating activity in cell-free extracts of *R. solani*. A preliminary report has been presented [6].

RESULTS AND DISCUSSION

Hydroxylation of phenylacetic acid by extracts of R. solani

Apparent phenylacetic acid-dependent O_2 consumption was detected with cell-free extracts from mycelia of *R. solani* isolates no. 22 (27 nmol/min/ml) and no. 450 (14 nmol/min/ml). No activity was detected in culture filtrates, indicating that hydroxylating ability is only intracellular. On fractionation of the mycelial extract with ammonium sulphate, the precipitate at 35–60% saturation possessed about a 3-fold greater sp. act. (5·8 nmol/min/mg protein) than the crude extract (1·85 nmol/min/mg protein) with a recovery of 82%. An attempt to purify the enzyme further was made by subjecting the active fraction (40 ml) to gel filtration on a column of Sephadex G 100. The active fraction (Ve; 85 ml) showed a final purification of protein activity of about 17-fold (32·3 nmol/min/mg protein). Further chromatography of the eluate from the Sephadex G 100 on DEAE-Sephadex A 50 with a linear gradient of NaCl (0–2 M) did not result in any fractions with hydroxylation activity. Further purification of this enzyme was not possible because of its instability.

* Part 4 in the series "Pathochemical Studies on Rhizoctonia Disease". For part 3 see Kohmoto, K. (1973) *J. Fac. Agric. Tottori Univ.* **8** 21.

Substrate specificity in aromatic hydroxylation

Because of instability problems, experiments with the enzyme were done using crude extracts or partially purified preparations. The substrate specificity of a crude preparation clearly indicated that the hydroxylase is specific for phenylacetic acid. Benzoic, phenylpropionic, *trans*-cinnamic, and phenoxyacetic acids were not oxidized; accelerated O_2 uptake began only when phenylacetic acid was added to reaction mixtures containing these aromatic acids. The substrate specificity coincided with that of the aromatic hydroxylation activity displayed by growing cells of *R. solani* [3].

Factors affecting the hydroxylation of phenylacetic acid

Optimum activity in 0·1 M Tris–acetate buffers occurred between pH 5 and 6. There was little activity at pH 4 or 8. The following experiments were done with 0·1 M Tris–acetate buffer at pH 5·2. The effect of NADH and NADPH as cofactors for the hydroxylase activity of gel-filtered extract was investigated. Without tetrahydrofolate, there was little O_2 consuming activity with either cofactor, whereas with tetrahydrofolate both cofactors were effective. Involvement of tetrahydropteridines in enzymatic hydroxylation has been reported for phenylalanine-4-hydroxylase from rats [7] and *trans*-cinnamic acid-4-hydroxylase from spinach leaves [8] and Alaska pea seedlings [9]. Phenylacetic acid-dependent O_2 consumption was found to be much greater in the presence of NADH than in the presence of NADPH. Addition of FAD and/or FMN to the reaction mixture caused a rapid increase of O_2 consumption which was not coupled to the hydroxylation of phenylacetic acid.

Na-diethyldithiocarbamate (10^{-3} and 10^{-4} M) and oxine (10^{-3} M) completely inhibited the phenylacetic acid-dependent O_2 consumption by the gel-filtered extract and *o*-phenanthroline (10^{-3} M) partially inhibited it; phenylthiourea and Na-EDTA (10^{-3} M) did not affect the reaction. On the contrary, α,α'-dipyridyl stimulated apparent O_2 consumption about 3-fold, although it is not clear that the enhanced reaction was associated with hydroxylation. Analogous stimulation by α,α'-dipyridyl has been reported for soluble NADH oxidase from *Bacillus subtilis* spores [10].

The rate of substrate-dependent O_2 consumption was directly proportional to the amount of enzyme in the reaction mixture. The K_m value for phenylacetic acid using partially purified hydroxylase was estimated to be $1{\cdot}2 \times 10^{-2}$ M, according to Lineweaver–Burk plots of substrate concentration vs hydroxylase activity. This K_m value is rather high, and suggests that a mechanism such as cellular compartmentalization of the substrate is required to explain the rapid biosynthesis of *m*-hydroxyphenylacetic acid by growing mycelia of *R. solani* [2]. In any case, these results provide the first evidence for enzymatic activity associated with the *meta*-specific hydroxylation of phenylacetic acid by *R. solani*. We tentatively propose to call this enzyme phenylacetic acid *meta* hydroxylase, because of its high substrate-specificity, and its requirement for NADH and molecular oxygen. Since *meta*-specificity is a rare phenomenon in the biological or chemical hydroxylation of aromatic acids [4,11], the characteristics of the hydroxylase are of great importance.

EXPERIMENTAL

Preparation of cell-free extracts. R. solani (Isolate nos. 22 and 450, cultural type IIIA *sensu* Watanabe and Matsuda [12]) was grown in stationary culture at 28° for 10 days in a modified Richards' medium supplemented with 0·5% polypeptone. Mycelial mats were collected on filter paper and washed ×3 with H_2O. The washed mats were suspended in 2 vol. of 67 mM Pi buffer at pH 6·5 which contained 1% Triton X-100 and 0·1% 2-mercaptoethanol. The mats were homogenized at 0° for 1 min and cellular debris was removed by centrifuging at 10000 *g* for 60 min. The supernatant was used as crude cell-free extract.

Partial purification. All operations were performed at 0–3°, except where noted. Differential precipitation of protein was done by successively treating crude extract or one of the resulting supernatants with $(NH_4)_2SO_4$ for 3 hr, followed by centrifugation at 10000 *g* for 60 min. The ppts were dissolved in a small vol. of 67 mM Pi buffer (pH 6·5) containing 0·1% 2-mercaptoethanol. The protein that precipitated between 35 and 60% saturation was applied to a Sephadex G 100 column (2·5 × 35 cm, Vo: 56 ml). Fractions (4·5 ml) were assayed for hydroxylating activity and for protein.

Protein assay. The Lowry method [13] was used to determine protein. Bovine serum albumin was used as a standard in the assay.

Measurement of enzyme activity. The standard reaction mixture (3 ml) contained 0·5 ml enzyme soln, 2·1 ml 67 mM Pi buffer (pH 6·5) or Tris–acetate buffer (pH 5·2) plus 0·1% 2-mercaptoethanol, 0·1 ml 20 mM tetrahydrofolate, 0·1 ml 10 mM NADH, and 0·2 ml 0·1 M substrate. After an equi-

libration period at 30°, the rate of O_2 consumption was determined with an O_2 electrode on addition of the compounds under test. All the substrates used were as the Na salts.

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