INVOLVEMENT OF PROTOCATECHUIC ACID IN THE METABOLISM OF PHENYLACETIC ACID BY *ASPERGILLUS NIGER*

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1. Introduction

Phenylacetic acid is usually metabolized via homogentisic acid [1–3] or homoprotocatechuic acid [4, 5]. However, preliminary studies in our laboratory have shown that *o*- and *m*-hydroxy phenylacetic acids, *m*-hydroxy benzyl alcohol, *m*-hydroxybenzoic acid and protocatechuic acid but not homoprotocatechuic acid and homogentisic acid are the metabolites that accumulate in the culture filtrates of a strain of *Aspergillus niger*, UBC 814 grown in the presence of phenylacetic acid. The present results indicate that a new pathway is operating in this strain.

2. Materials and methods

Aspergillus niger (UBC 814) was grown for 40 hr at 30° on a synthetic medium [6] supplemented with 0.1% (w/v) phenylacetic acid. Replacement studies were carried out with washed mycelial mats placed in solutions containing 0.5 mg/ml of different substrates in 0.025 M sodium phosphate buffer, pH 7 (except in the case of *m*-cresol where 0.1 mg/ml was used). The replacement cultures were kept on a rotary shaker at 30° for 6 hr and the phenolic compounds formed were analysed.

2.1. Analysis of phenolic compounds

The cultures were filtered through glass wool, the medium was acidified to pH 2 with 2 N HCl and extracted repeatedly with diethyl ether. The combined ether layers were concentrated under vacuum and shaken with 5% $NaHCO_3$. The organic layer (neutral fraction) was removed and the aqueous layer, after acidification to pH 2, was extracted with ether. From the ether layer the *o*-dihydroxy acids were separated as their lead salts by shaking with 5% lead acetate. The free acids were obtained by decomposing the salts with hydrogen sulfide and taken in ether (*o*-dihydroxy acid fraction). The ethereal layer containing the remaining acids (acidic fraction), *o*-dihydroxy acid fraction and the neutral fraction were concentrated to a small volume and subjected to paper chromatography on Whatman No. 3 filter paper using the following solvent systems:

A: isopropanol–ammonia–water (20:1:2, v/v),
B: benzene–acetic acid–water (10:7:3, v/v, organic phase) and
C: formic acid–water (2:98).

Phenolic compounds were located by UV light and also by spraying either with diazotized *p*-nitroanaline followed by 1 N NaOH or with 1% ferric chloride–ferricyanide mixture (1:1). The products were identified by comparing their R_f values in different solvent systems and UV absorption spectra with the authentic compounds. The individual compounds were isolated from the paper chromatograms by eluting with hot ethyl acetate. UV spectra were taken in ethyl acetate using Unicam SP-700A recording spectrophotometer. Infra-red spectra were recorded in a Carl Zeiss Jena UR10 spectrophotometer.

Table 1
R_f values and λ_{max} of the phenolic compounds.

Name of the compounds	R_f values in solvent systems						λ_{max} (nm)	
	A		B		C			
	a	i	a	i	a	i	a	i
o-Hydroxy phenylacetic acid	0.52	0.52	0.43	0.44	0.88	0.88	274	274
m-Hydroxy phenylacetic acid	0.29	0.28	0.31	0.31	0.82	0.82	273	273
m-Cresol	0.92	0.91	0.96	0.96	–	–	273	273
m-Hydroxy benzyl alcohol	0.81	0.81	0.19	0.19	0.81	0.80	274	274
m-Hydroxy benzoic acid	0.25	0.25	0.35	0.34	0.70	0.71	295	295
Protocatechuic acid	0.03	0.03	0.03	0.03	0.57	0.57	259 292	259 292

a = Authentic sample; i = isolated products.

3. Results

The neutral fraction contained only one aromatic compound, which has the same R_f value as that of *m*-hydroxybenzyl alcohol in different solvent systems (table 1). The UV spectrum of the product had λ_{max} at 274 nm with shoulders at 269 and 280 nm. The IR spectrum of the isolated compound had the following absorption:

3425 and 1050 cm^{-1} (primary –OH)
3100 and 1170 cm^{-1} (phenolic –OH)
1600 cm^{-1} (aromatic ring) and 800 cm^{-1} (*m*-substitution).

The IR spectrum of the compound was superimposable on that of authentic *m*-hydroxy benzyl alcohol.

The *o*-dihydroxy acid fraction also contained only one compound; the R_f value and the λ_{max} (table 1) of which were identical with those of authentic protocatechuic acid. Neither homoprotocatechuic acid nor 2,3-dihydroxy benzoic acid was detected.

The acidic fraction was analyzed by uni- and two dimensional chromatography. When chromatograms were developed with solvent A and B there appeared two spots one of which was found to be *o*-hydroxy phenylacetic acid. The other spot was found to be a mixture of two compounds which got separated well in solvent C. They were identified as *m*-hydroxy phenylacetic acid and *m*-hydroxy benzoic acid. The identity of these compounds were further confirmed by running two dimensional chromatograms using solvent systems A/B and B/C.

3.1. Analysis of the replacement culture media

Phenylacetic acid replacement culture medium contained *o*- and *m*-hydroxy phenylacetic acids as evidenced by chromatography and UV spectra, whereas the presence of both *p*-hydroxy phenylacetic acid and mandelic acid could not be detected. Replacement with *m*-hydroxy phenylacetic acid yielded only a neutral product, which had λ_{max} at 273 nm with shoulders at 267 and 280 nm. The R_f values of this compound in solvent systems A and B were same as those of *m*-cresol. Even traces of homoprotocatechuic acid or homogentisic acid could not be detected.

m-Cresol replacement culture medium contained predominantly a neutral product which was found to be *m*-hydroxy benzyl alcohol judged by its R_f values in different solvent systems and UV spectrum. When *m*-hydroxy benzyl alcohol was used in the replacement

medium, the only product formed was identified as *m*-hydroxy benzoic acid. However, when *m*-hydroxy benzaldehyde was used as the substrate, equal amounts of alcohol and acid were formed. The formation of *m*-hydroxy benzyl alcohol indicated the reversibility of the alcohol to aldehyde reaction.

Replacement with *m*-hydroxy benzoic acid yielded only one compound which was identified as protocatechuic acid. Gentisic acid was not detected.

These results indicate the operation of the following new pathway for the degradation of phenylacetic acid in this organism:

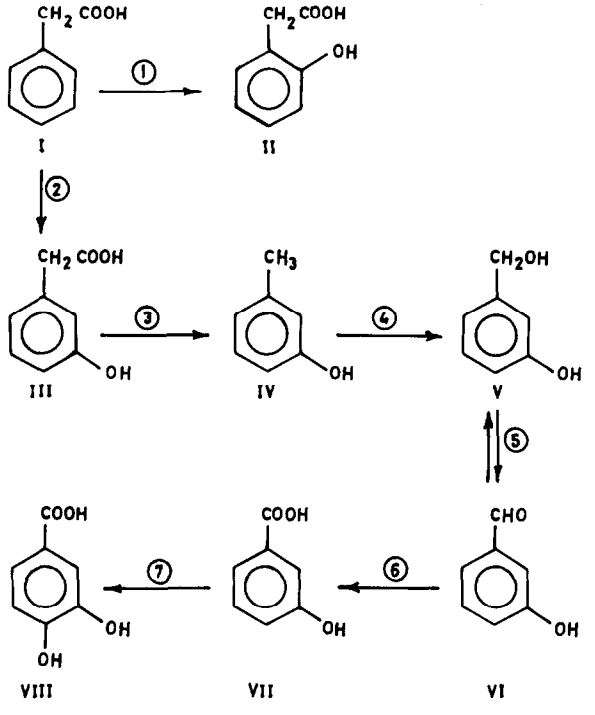

Fig. 1. Proposed scheme for the degradation of phenylacetic acid by *Aspergillus niger* (UBC 814). I. Phenylacetic acid. II. *o*-Hydroxy phenylacetic acid. III. *m*-Hydroxy phenylacetic acid. IV. *m*-Cresol. V. *m*-Hydroxy benzyl alcohol. VI. *m*-Hydroxy benzaldehyde. VII. *m*-Hydroxy benzoic acid. VIII. Protocatechuic acid. 1: Phenylacetic acid 2-hydroxylase; 2: phenylacetic acid 3-hydroxylase; 3: *m*-hydroxy phenylacetic acid decarboxylase; 4: *m*-cresol hydroxylase; 5: *m*-hydroxy benzyl alcohol dehydrogenase; 6: *m*-hydroxy benzaldehyde dehydrogenase; 7: *m*-hydroxy benzoic acid 4-hydroxylase.

In order to get more insight into the metabolism of phenylacetic acid in this organism, attempts were made to isolate the different enzymes involved in this pathway. In cell-free extracts, prepared from cultures grown on medium containing phenylacetic acid, the presence of *m*-hydroxy benzyl alcohol dehydrogenase, *m*-hydroxy benzaldehyde dehydrogenase and *m*-hydroxy benzoic acid 4-hydroxylase could be demonstrated. *m*-Hydroxy benzyl alcohol dehydrogenase has been partially purified by protamine sulfate and DEAE-cellulose treatments. This enzyme showed an absolute requirement for NADP. A similar observation has been reported by Forrester and Gaucher [7]. *m*-Hydroxy benzaldehyde dehydrogenase required NAD as the cofactor in cell-free systems. *m*-Hydroxy benzoic acid 4-hydroxylase has also been partially purified according to the method of Premkumar et al. [8]. This enzyme required NADPH and FAD for its activity.

The bacterial break-down of phenylacetic acid generally occurs either by the homogentisic pathway [1, 3] or by the homoprotocatechuic acid pathway [4, 5]. However, not much information is available on the fungal degradation of phenylacetic acid. Benzaldehyde has been characterized as a product of phenylacetic acid oxidation by *Penicillium chrysogenum* [9]. The first step in the microbial degradation of phenylacetic acid appears to be its hydroxylation at the *ortho* or the *para* position. The *o*-hydroxy phenylacetic acid is converted to homogentisic acid whereas *p*-hydroxy phenylacetic acid gives rise to homoprotocatechuic acid. In an alternative pathway suggested by Crowden to be operative in the fungus *Polyporus tumulosus*, *p*-hydroxy phenylacetic acid is apparently converted to *p*-hydroxy mandelic acid [10]. *o*-Hydroxy phenylacetic acid and homogentisic acid were found to be the main metabolites of phenylacetic acid in *A. niger*, the *m*- and *p*-hydroxy phenylacetic acids being the minor products [11]. However, Faulkner and Woodcock [12] have reported the absence of homogentisic acid in the culture filtrates of *A. niger* grown on phenylacetic acid. This disparity may however, be attributed to the difference in the strains of *A. niger* used by the two groups of workers. This is not surprising, as striking differences in the pathway of phenylacetic acid have also been reported in two strains K_1 and K_2 of *P. fluorescens* by Kunita [1, 4]. Our results with the strain UBC 814 of *A. niger* represent the first report on the in-

volvement of *m*-hydroxy benzyl alcohol and protocatechuic acid in phenylacetic acid oxidation and serve to show the multiplicity of pathways available for the catabolism of phenylacetic acid not only in different organisms but also in different strains of the same organism.

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