p-HYDROXYMANDELIC ACID - A KEY INTERMEDIATE IN THE METABOLISM OF DL(±)-PHENYLALANINE BY ASPERGILLUS NIGER

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Summary: The metabolism of phenylalanine by a strain of Aspergillus niger, isolated from the soil by enrichment culture has been studied. Analyses of the culture filtrates and replacement studies with various metabolites have revealed the operation of a degradative pathway involving p-hydroxymandelate as a key intermediate in this organism. p-Hydroxymandelate has been isolated from the cultural filtrates and its identity established by UV, IR and chromatographic techniques. A scheme for the degradation of phenylalanine in this organism has been proposed.

1. Introduction

The bacterial degradation of phenylalanine usually proceeds by its conversion to either tyrosine or phenylpyruvate (1,2). In contrast to the bacterial systems, the fungal metabolism of phenylalanine is initiated, in most cases, by its conversion to cinnamate (1,2). Tyrosine and phenylpyruvate are further metabolised by either homoprotocatechuate pathway or homogentisate pathway, while cinnamate is converted to protocatechuate through benzoate (1,2). Among the other pathways suggested for the phenylalanine metabolism is the one involving p-hydroxymandelate (3,4). However, a clearcut evidence in this direction is lacking. The present paper deals with the isolation and identification of p-hydroxymandelate as a key intermediate in the metabolism of phenylalanine by Aspergillus niger.

2. Methods

2.1 Cultivation

A strain of Aspergillus niger, isolated from the soil by enrichment culture technique, was grown on a synthetic medium (5) supplemented with 0.1% (W/V) DL-phenylalanine as the inducer, at 30°. Stock cultures were maintained on slants of the same medium solidified with 2% (W/V) agar.

2.2 Replacement studies

Mycelial felts of A. niger, during various phases of growth, were taken, washed with water and suspended in solutions containing 1 mg/ml of different substrates in 0.025 M sodium phosphate buffer, pH 7.0. The culture suspensions were placed in a rotary shaker at 30° for 4 to 6 hours and the products formed were analysed.

2.3 Analysis of aromatic compounds

The culture filtrates were acidified to pH 2 with 2 N HCl and extracted repeatedly with diethyl ether. The ether extracts were pooled and the combined ether extract was resolved into the acidic and neutral fractions using 5% $NaHCO_3$. The two fractions were dried over anhydrous sodium sulfate, concentrated and subjected to chromatography on Whatman No.3 filter paper using the following solvent systems:

A. benzene - acetic acid - water (10:7:3, V/V, organic phase)

B. formic acid - water (2:98, V/V)

C. isopropanol - ammonia - water (20:1:2, V/V)

The aromatic compounds were located on the chromatograms by UV light. Carboxylic acids were detected by spraying

Table I

R_f Values and λ_{max} of the phenolic compounds

Name of the compound	R_f values in solvent systems A		B		C		λ_{max} (nm)	
	a	i	a	i	a	i		
o-Hydroxyphenyl acetic acid	0.46	0.46	0.88	0.88	0.52	0.51	274	274
p-Hydroxyphenyl acetic acid	0.30	0.31	0.82	0.83	0.42	0.43	277 284	277 284
p-Hydroxymandelic acid	0.01	0.01	0.89	0.88	0.23	0.23	276 282	276 282
p-Hydroxybenzoic acid	0.36	0.35	0.60	0.60	0.35	0.36	254	254
Procatechuic acid	0.02	0.03	0.58	0.59	0.03	0.02	259 292	259 292
p-Hydroxybenzaldehyde	0.40	0.40	0.58	0.58	0.38	0.37	268	268

a = Authentic sample; i = isolate products

with aqueous potassium permanganate. The phenolic compounds were spotted by spraying either with diazotised p-nitroaniline followed by alkali or with 1% ferric chloride - ferricyanide mixture (1:1). Carbonyl compounds were recognised by their colored products with 2,4-dinitrophenylhydrazine.

The products were identified by comparing their R_f values in different solvent systems and UV spectra in ethyl acetate, with those of the authentic samples. The individual compounds were eluted from the paper chromatograms with ether. UV spectra were recorded in Unicam SP 700 A spectrophotometer. IR spectra were recorded in a Carl Zeiss Jena UR 10 spectrophotometer by nujol mull technique.

3. Results and Discussion

3.1 Analysis of culture filtrates

The neutral fraction contained a single, phenolic, 2,4-dinitrophenylhydrazine positive compound which could be identified as p-hydroxybenzaldehyde. The acidic fraction, by two dimensional chromatographic analysis employing solvent systems A/B and C/A, could be resolved into five components which were identified as o- and p-hydroxyphenylacetic acids, p-hydroxymandelic acid, p-hydroxybenzoic acid and protocatechuic acid. The UV spectra of each of the above five compounds corresponded well with those of the authentic samples (Table I). From the culture filtrates obtained after 30 hours of growth, p-hydroxymandelic acid was isolated by preparative paper chromatography using solvent system A. The isolated material showed major IR absorptions at

a. 3200-3500 cm^{-1} (broad band) Hydrogen bonded and multiple hydroxyl groups.

Table II

Results of Replacement Experiments

Compounds added to the replacement medium	Phenolic compounds formed					
	o-Hydroxyphenyl acetic acid	p-Hydroxyphenyl acetic acid	p-Hydroxymandelic acid	p-Hydroxybenzaldehyde	p-Hydroxybenzoic acid	Protocatechuic acid
Phenylalanine	+++	+++	+	+	+++	++
Phenylpyruvic acid	+++	+++	+	+	+++	++
Phenylacetic acid	+++	+++	++	+	+++	++
o-Hydroxyphenyl acetic acid	+++	-	-	-	-	-
p-Hydroxyphenyl acetic acid	-	+++	+++	+	++	+++
p-Hydroxymandelic acid	-	-	+++	+	+++	+++
p-Hydroxybenzaldehyde	-	-	-	+++	+++	+++
p-Hydroxybenzoic acid	-	-	-	-	+++	+++
Protocatechuic acid	-	-	-	-	-	+++

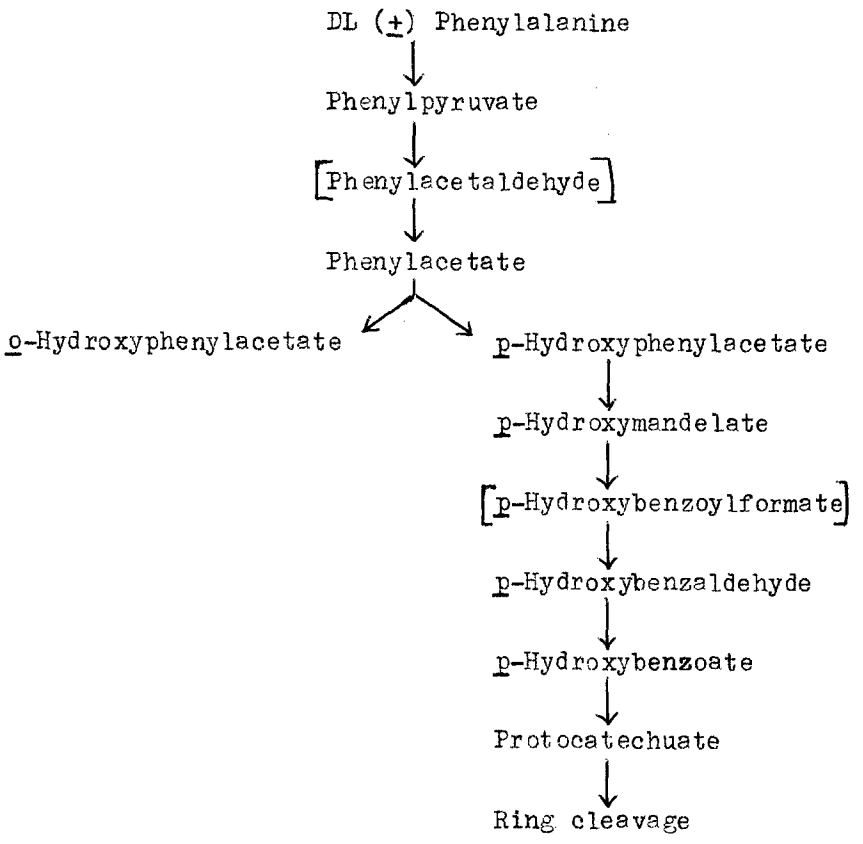

Fig.1 Proposed pathway for phenylalanine dissimilation by Aspergillus niger.

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b. 2850-2950 cm^{-1} (broad band) Aromatic C-H's and COOH.

c. 1700, 1460, 1360 cm^{-1} COOH group

d. 1605 cm^{-1} Aromatic C=C

e. 1260-1270 cm^{-1} Phenolic O-H and COOH group

f. 1170 cm^{-1} Phenolic O-H

g. 1080 cm^{-1} Secondary alcohol, and

h. 840 cm^{-1} Aromatic 1,4-disubstitution.

The IR spectrum was superimposable on that of the authentic p-hydroxymandelic acid.

3.2 Replacement culture analysis

The phenolic acids which accumulated in the culture filtrates, when the washed mycelial felts of A. niger were incubated with the various metabolites of phenylalanine have been shown in Table II.

The foregoing results serve to illustrate the existence of the following scheme for the metabolism of phenylalanine in A. niger (Fig.1).

In order to get more insight into the mechanism of phenylalanine dissimilation, attempts were made to isolate the individual enzymes of the metabolic sequence. Cell-free extracts of A. niger grown on phenylalanine contained both amino acid oxidase and L-phenylalanine: α-ketoglutarate transaminase activities, fully confirming the participation of phenylpyruvate in the metabolism. Neither phenylalanine--ammonia lyase nor phenylalanine hydroxylase activities could be detected. Phenylpyruvate was oxidised to phenylacetate by crude extracts. The intermediary formation of phenylacetaldehyde in this reaction is yet to be established. The direct conversion of phenylpyruvate to o- and p-hydroxyphenylacetates did not occur; it was however possible to demonstrate the conversion of phenylacetate to o- and p--hydroxyphenylacetates. NADPH participates in this reaction.

The further metabolism of o-hydroxyphenylacetate is under investigation. The available data suggests that it is not metabolised by A. niger. On the other hand, p-hydroxyphenylacetate is rapidly metabolised by the whole cells to p-hydroxymandelate. p-Hydroxymandelate dehydrogenase activity could be demonstrated in the particulate as well as the

soluble fractions, $NADP^+$ being the most effective cofactor in both the systems. p-Hydroxybenzoate hydroxylase and protocatechuate dioxygenase activities have also been detected. The above results are in conformity with the postulated pathway.

Although p-hydroxymandelate is a naturally occurring compound (6), not much information is available on its biosynthesis. Perrin and Towers (3) have detected radioactive p-hydroxymandelate in the culture filtrates of Polyporous hispidus grown on phenylalanine or tyrosine. On the basis of 2,5-dihydroxybenzoylformate formation from both p-hydroxymandelate and p-hydroxyphenylacetate, Crowden (4) has suggested that p-hydroxymandelate is formed from p-hydroxyphenylacetate. Bhat et al. (7) have isolated an inducible enzyme from P. convexa which converts mandelate to p-hydroxymandelate. Jamaluddin et al. (8) in their studies with A. niger (UBC 814) grown on mandelate have shown the conversion of p-hydroxymandelate to p-hydroxybenzoate, which is the converging point in the oxidative metabolism of mandelate and p-hydroxymandelate. They were, however, unable to demonstrate the conversion of mandelate to p-hydroxymandelate.

Our results with A. niger strain, isolated from the soil, constitute the first report on the isolation and identification of p-hydroxymandelate as a key metabolite of phenylalanine catabolism. Except for a single dichotomy at the phenylacetate stage, all available evidence indicates a total absence of alternative metabolic routes in contrast to the results of Perrin and Towers (3), where the cinnamate pathway predominates.

It is of interest to note that the organism converts

p-hydroxyphenylacetate only to p-hydroxymandelate and neither to homoprotocatechuate nor to homogentisate, although such pathways are known to provide the major routes for its metabolism (1,2,9). Such observations lead to a very important and a fundamental question: what are the factors governing the choice of any metabolic pathway in preference to others, even if the latter cases require a lesser complement of enzymes? With such an end in view, attempts are being made to study in detail, the individual enzyme reactions of the pathway.

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