Note

Degradation of Homogentisic Acid by *Aspergillus fumigatus* ATCC 28282

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Many studies on the formation of homogentisic acid (2,5-DiOHPA) from tyrosine,^{1~3)} phenylalanine^{4~7)} and phenylacetic acid^{8~10)} by microorganisms have appeared in the literature. Two degradative pathways for 2,5-DiOHPA in microorganisms have been described: (i) the gentisic acid pathway¹¹⁾ and (ii) the oxidative ring cleavage pathway.¹²⁾

However, little information is available on the fungal degradation of 2,5-DiOHPA in contrast to the many investigations with bacteria. Although 2,5-DiOHPA was oxidized directly to ring-cleaved products by *A. niger* during sporulation,^{6,13)} it is not certain whether the same reactions occur when other species of *Aspergillus* oxidize 2,5-DiOHPA.

In a previous paper,⁷⁾ we reported the formation of 2,5-DiOHPA as a key intermediate in the metabolism of phenylalanine and tyrosine by *A. fumigatus* ATCC 28282. This note describes the gentisic acid pathway for the

degradation of 2,5-DiOHPA by *A. fumigatus* ATCC 28282.

The conidia of *A. fumigatus* ATCC 28282 were inoculated on a medium containing 2.5% glucose, 2.0% monosodium glutamate·H₂O, 0.5% KH₂PO₄ and 0.2% MgSO₄·7H₂O in 300-ml Erlenmeyer flasks (150 ml per flask) for 7~10 days at 30°C without shaking. The mycelial mats were washed once with sterile distilled water and reincubated at 30°C for 3~5 days with an aqueous solution of the substrate (0.01% 2,5-DiOHPA, pH 6.0) dissolved in 0.2M disodium phosphate-0.1M citric acid buffer (100 ml per flask). Under these conditions, 2,5-DiOHPA was not converted to gentisic acid and gentisaldehyde in control experiments in which mycelial mats were omitted.

For the detection and identification of the intermediary products of 2,5-DiOHPA degradation, the culture medium was acidified to pH 4.0 with H₂SO₄ and extracted with ethyl acetate. The ethyl acetate extract was concentrated and subjected to thin-layer chromatography (TLC) on silica gel plates (200 nm, 20×20 cm, without fluorescence, Tokyo Kasei). The plates were developed in 3 solvent systems: A, chloroform-ethyl acetate-formic acid (50:10:2.5, v/v); B, benzene-dioxane-acetic acid (90:20:4, v/v); and C, hexane-ethyl ether (30:70, v/v). The metabolites were detected on TLC plates by the fluorescence under ultraviolet light and by spraying Folin-Ciocalteu reagent (PR)¹⁴⁾ (Table I), and identified by mass spectrometric analysis.

The bands of metabolites A and B (Table I) were separately scraped off from the TLC plates and eluted from the silica gel with ethyl ether. Each concentrated ether solution was introduced directly into the ionization chamber (270°C) of a mass spectrometer (Shimadzu-LKB-9000) at 70 eV energy. The mass spectra of metabolites A and B were identical with those of authentic gentisic acid and gentisaldehyde.

These facts were confirmed by the use of radio tracer methodology. Radioactive 2,5-DiOHPA was prepared

TABLE I. THIN-LAYER CHROMATOGRAPHY OF FUNGAL DEGRADATION
PRODUCTS OF HOMOGENTISIC ACID

Compounds	R _f Values ^a			Detection ^b	
	Soln. A	Soln. B	Soln. C	D ₁	D ₂
Authentic					
Gentisic acid	0.50	0.52	0.08	Dark blue	Blue fluorescence
Gentisaldehyde	0.67	0.78	0.64	Dark blue	Dark yellow
Metabolites					
A	0.50	0.52	0.08	Dark Blue	Blue fluorescence
B	0.67	0.78	0.64	Dark blue	Dark yellow

^a Soln. A, chloroform-ethyl acetate-formic acid = 50:10:2.5 (v/v); Soln. B, benzene-dioxane-acetic acid = 90:20:4 (v/v); Soln. C, hexane-ethyl ether = 30:70 (v/v).

^b D₁, Folin-Ciocalteu reagent; D₂, ultraviolet light.

microbially from DL-[3-¹⁴C]-tyrosine (50 μ Ci, New England Nuclear) according to Yoshizako *et al.*,⁷⁾ and the radioactive 2,5-DiOHPA was separated by TLC with solvent A (*R_f*=0.14). The band corresponding to authentic 2,5-DiOHPA was scraped off from the plates and eluted from the silica gel with 20 ml of distilled water. The specific radioactivity of 2,5-DiOHPA (9×10^7 dpm/mmol 2,5-DiOHPA) in this solution was the same as that of tyrosine (8.3×10^7 dpm/mmol tyrosine). Twenty ml of the eluate was added to 480 ml of 0.2 M disodium phosphate–0.1 M citric acid buffer containing 0.15 g of cold 2,5-DiOHPA (7.5×10^5 dpm/500 ml, pH 6.0). The mixture was used as a replacement culture medium (100 ml per flask). After cultivation (30°C, 3 days) and treatment of the culture filtrate by the same method as that described in non-radioactive experiments, the concentrated ethyl acetate extract (20 μ l, 2.3×10^4 dpm) was spotted onto a TLC plate. The TLC plate was developed in solvent B. After detection of the presence of gentisic acid and gentisaldehyde by the fluorescence under ultraviolet light and by spraying with PR, each spot was scraped off from the TLC plate. The radioactivity of each spot was measured with a liquid scintillation counter (TRI CARB 3380). It was found that the radioactivity of ¹⁴C-labelled 2,5-DiOHPA was incorporated into the transiently accumulated gentisic acid (290 dpm) and gentisaldehyde (65 dpm) per plate.

Gentisic acid was also isolated from the culture fluid in which washed mycelial mats had been incubated at 30°C for 3 days with gentisaldehyde (0.01%, pH 6.0) dissolved in 0.2 M disodium phosphate–0.1 M citric acid buffer (100 ml per flask). The melting point, and the ultraviolet, infrared (KBr) and mass spectra of this compound were essentially the same as those of authentic gentisic acid.

The data obtained in this study show that *A. fumigatus* ATCC 28282 clearly degrades 2,5-DiOHPA to gentisic acid *via* gentisaldehyde.

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