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# ROLE OF CINNAMATE IN BENZOATE PRODUCTION IN *PENICILLIUM BREVICOMPACTUM*

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**Key Word Index**—*Penicillium brevicompactum*; Deuteromycete; biosynthesis; benzoate; phenylalanine ammonia-lyase.

**Abstract**—Radioisotope feeding experiments with solid cultures of *Penicillium brevicompactum* demonstrate that cinnamate is an intermediate in the conversion of L-phenylalanine to benzoate. The first enzyme in this pathway, phenylalanine ammonia-lyase, has been purified 62-fold from surface liquid cultures.

## INTRODUCTION

The imperfect fungus, *Penicillium brevicompactum*, produces two sets of benzoic acid derivatives: (a) the pebrolides, three sesquiterpenoid benzoates [1], and (b) *N*-benzoyl-L-phenylalanine, *N*-benzoyl-L-phenylalaninol [2] and the ester formed between the latter two amides, asperphenamate [2, 3]. We have already shown that all the carbon atoms of the second set of compounds are derived from L-phenylalanine [2, 3] and have demonstrated that in solid culture the appearance of asperphenamate correlates with the development of an aerial mycelium [4]. Solid cultures that are precluded from forming an aerial mycelium by being grown between two sheets of cellophane do not form the 'special metabolite' [5] until such time as that preclusion is lifted [4]. Formation of a conidial head on aerial hyphae is not a prerequisite for asperphenamate synthesis since a class of developmental mutants that produces an aerial mycelium but does not form penicilli yields normal amounts of asperphenamate (Campbell, I. M., *et al.*, unpublished work). Prior to attempting to exploit the asperphenamate system as a model for special metabolite gene expression, some biosynthetic questions needed to be resolved. In this report we examine the first stage of benzoate production.

The most likely first step in the conversion of phenylalanine to benzoate is the production of cinnamate. The phenylalanine → cinnamate conversion is well known in both plants and micro-organisms [6, 7] although it has not yet been demonstrated in the Deuteromycetes. If this step does occur in *P. brevicompactum*, it should be possible to demonstrate (a) that radioisotope, flowing from L-phenylalanine to benzoyl groups, can be trapped in cinnamate; (b) that radioisotope administered in cinnamate can be transferred efficiently to the benzoate unit; and (c) that phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) activity is present in the fungal thallus. In this paper we confirm these three predictions.

## RESULTS AND DISCUSSION

In all the work we used a mutant strain of *P. brevicompactum* (ATCC 9056) that is blocked in a late stage of conidiation. Thus, on solid culture the mutant (MG 78-14) forms an abundant aerial mycelium which gives rise to morphologically normal penicilli. However, whereas each sterigmatum of wild type *P. brevicompactum* yields many scores of green conidia, each sterigmantum of mutant MG 78-14 yields only a few white conidia (ten or less). Mutant MG 78-14 synthesizes the full special metabolite spectrum of its wild type parent: mycophenolic acid, the brevianamides and the members of the asperphenamate group. However, the biomass of mutant MG 78-14 is more easily and more efficiently disrupted in a French press (based on total protein released per unit weight of biomass) and the resulting cell free extracts are significantly lower in protease activity than those from wild type *P. brevicompactum*.

In our first experiment, L-[U-$^{14}$C]phenylalanine was fed to eight 42-hr-old 'over' cultures [8] of mutant MG 78-14. Cultures of such an age have developed a rich aerial mycelium and are in the first stages of penicillus formation. Following a 4-hr incubation period, the biomass was freed from the solid growth medium and was extracted with chloroform. Dilution analyses were performed on aliquots of this extract with unlabelled cinnamate and benzoate; incorporation values [9] of 0.17 and 2.01%, respectively, were recorded. These data establish that accessible pools of both cinnamate and benzoate exist in the organism and that both acids are derived metabolically from phenylalanine.

[3-$^{14}$C]Cinnamate was next fed to eight 42-hr-old 'over' cultures. Following a 6-hr incubation, dilution analyses were performed with unlabelled cinnamate, benzoate, *N*-benzoyl-L-phenylalaninol and asperphenamate. The incorporation values recorded from these four analyses were 0.05, 12.3, 0.26 and 0.86%, respectively. Collectively, these data establish a role for cinnamate in the biosynthesis of benzoate and benzoyl groups in *P. brevicompactum*.

Further support for a role for cinnamate was obtained at the enzymological level. Cell-free extracts were prepared from 42-hr-old cultures of mutant MG 78-14 using a French press. These extracts were assayed for phenylalanine ammonia-lyase (PAL). In twenty independent assays, an average specific activity of 1.1 ± 0.5 mU/mg of

total protein was recorded. From the physiological point of view, Table 1 shows an even more significant finding. When 96-hr-old 'between' cultures [8] of mutant MG 78-14 were stripped and the PAL level was monitored as a function of culture age in the 'stripped-between' format, these levels were seen to build in parallel with the development of an aerial mycelium and the appearance of asperphenamate and of its component parts. Table 1 also indicates that levels of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) decrease as aerial hyphae emerge.

Using biomass produced from 52-hr-old surface liquid cultures of mutant MG 78-14, i.e. cultures that are at approximately the same stage of development as were the 42-hr-old 'over' cultures used in the isotope incorporation work, the PAL activity was purified 62-fold using ion exchange and gel permeation chromatography (Table 2). The enzyme has a pH optimum around 8.3. Presently we are attempting to purify PAL to homogeneity.

The consensus of the findings described above is that the first step in the production of benzoate in *P. brevicompactum* is the conversion of phenylalanine to cinnamate. It remains to be seen if the cinnamate → benzoate conversion parallels fatty acid β-oxidation [10] or proceeds through benzaldehyde without the involvement of coenzyme A esters [11].

## EXPERIMENTAL

*Biological material.* The developmental mutant MG 78-14, derived by $^{60}$Co irradiation of *Penicillium brevicompactum* Bainer (ATCC 9056), was maintained on Czapek Dox-glucose/0.1% corn steep liquor slants at 23°. Subcultures were made every 3 weeks. Slants that were no more than 96 hr old were used to provide spore inocula for expts.

'Over' cultures were prepared with Sabouraud-glucose medium (1.5% agar) as described previously [8] using as inoculum an aliquot (0.1 ml) of a suspension of spores (*ca* $10^7$/ml) in 0.1% Tween 80 in distilled $H_2O$. 'Over' cultures were grown at 23° for 42 hr prior to use in isotope incorporation work.

Surface liquid cultures were grown on Sabouraud-glucose medium (300 ml) contained in Fernbach flasks (2.8 l.). The inoculum for 12–15 such flasks was prepared by homogenizing 3–4 slants in distilled $H_2O$ (400 ml) under sterile conditions and adding aliquots of the homogenate to the Fernbach flasks. Surface liquid cultures were grown for 52 hr at 23°.

*Isotope incorporation and dilution analyses.* (a) [U-$^{14}$C]Phenylalanine (80 μCi, 450 μCi/μmol, Research Products International, Mount Prospect, IL) in distilled $H_2O$ (1.6 ml) was sprayed *carefully* onto eight 42-hr-old 'over' cultures of *P. brevicompactum* mutant MG 78-14. A standard chromatograph sprayer that had a particularly coarse spray (obtained from Kontes Glass, Vineland, NJ) was used for this purpose. Following a 4-hr incubation period, the biomass (2.4 g, wet wt) was homogenized in $CHCl_3$ (10 ml). Particulate material was removed by filtration and aliquots (0.2 ml) were added separately to standard amounts of cinnamate (100.2 mg) and benzoate (97.8 mg) in $CHCl_3$. The cinnamic and benzoic acids were crystallized sequentially from aq. HOAc and EtOAc–isooctane several times until a constant specific activity resulted. From these specific activity data, incorporation values were calculated [9]. (b) [3-$^{14}$C]Cinnamate (24 μCi, 53 μCi/μmol, Research Products International) was sprayed onto eight 42-hr-old 'over' cultures of MG 78-14 and dilution analyses were performed with cinnamic and benzoic acids, with *N*-benzoyl-L-phenylalaninol and asperphenamate as described above.

*Release of PAL from* P. brevicompactum. In a typical expt, the biomass from fifteen 52-hr-old surface liquid cultures was culled by filtration and was washed briefly with ice-cold distilled $H_2O$

Table 1. PAL, glucose-6-phosphate dehydrogenase (GPD), asperphenamate (I), *N*-benzoylphenylalanine (II) and *N*-benzoylphenylalaninol (III) levels as a function of development in a 'stripped-between' culture of the *P. brevicompactum* mutant MG 14-78

| Age as a 'stripped-between' culture (hr)* | Levels of: PAL (mU/mg) | GPD (U/mg) | I† | II† | III† | Morphology |
|---|---|---|---|---|---|---|
| 0 | <0.01 | 3.77 | − | − | − | Vegetative mycelium only |
| 12 | 1.2 | 2.64 | + | + | + | Aerial mycelium present |
| 25 | 2.0 | 1.24 | + | + | + | Penicilli present |

*The 'between' cultures that were stripped were 96 hr old.
†Assayed qualitatively by TLC (silica gel, $C_6H_6$–dioxane–HOAc, 30:10:1).
+ = detected; − = absent.

Table 2. Partial purification of PAL from *P. brevicompactum*

| Step | Vol. (ml) | Protein amount (mg) | Specific activity (mU/mg) | Purification |
|---|---|---|---|---|
| Crude extract | 140 | 816 | 2.66 | 1 |
| Supernatant following protamine treatment | 135 | 622 | 2.85 | 1.1 |
| DEAE-Sepharose | 14.6 | 44.8 | 40.2 | 15.1 |
| Biogel A 1.5 | 34.1 | 9.4 | 165.0 | 62.0 |

before being pressed free of superficial $H_2O$ between layers of filter paper. Subsequent operations were conducted at 4°. The biomass (55.3 g pressed wet wt, still containing 86% residual $H_2O$) was homogenized for 1 min in 110 ml of the following freshly prepared 'TRISMEG' buffer (pH 7.9): tris-(hydroxylmethyl)aminomethane (100 mM), mercaptoethanol (3 mM), ethylenediamine tetraacetic acid (1 mM) and glycerol (10%). The hyphal fragments so formed were further disrupted in a French pressure cell under 6000 psi. A cell-free extract was prepared from these pressings by centrifugation at 12000 *g* for 20 min. Typically such extracts contained 816 mg of total protein as determined by the method of Bradford [12]. The release of PAL from 'over' culture biomass was handled similarly.

*Assays for PAL.* (a) Where extracts contained significant amounts of UV-absorbing material, aliquots (0.1–0.5 ml) were incubated for 30 min at 23° with [U-$^{14}$C]phenylalanine (20 $\mu$M, 0.25 $\mu$Ci) dissolved in 'TRISMEG' buffer (pH 8.3). The reaction was terminated with conc HCl (0.1 ml) and standard amounts (*ca* 100 mg) of cinnamate were added to the incubation mixture in $CHCl_3$ (5 ml). Following thorough mixing, the organic layer was removed and was freed of unconsumed phenylalanine by passage of the organic layer through a column (1 × 5 cm) of silica gel. The eluant was treated with charcoal and the cinnamate it contained was crystallized from $C_6H_6$–isooctane to a constant specific activity. From the latter, the rate of cinnamate production was calculated. (b) PAL is more conveniently measured spectrophotometrically by monitoring the rate of change of absorption at 270 nm of a solution containing an aliquot of the extract (0.1 ml) in 10 mM L-phenylalanine in 'TRISMEG' buffer (pH 8.3, 1.5 ml). $\varepsilon = 19351$.

Glucose-6-phosphate dehydrogenase was assayed according to the method of Kornberg and Horecker [13].

Units (U) of both PAL and glucose-6-phosphate dehydrogenase are expressed as $\mu$mol of product formed per min.

*Partial purification of PAL.* The cell-free extract (140 ml) derived above was treated with protamine sulphate (105 mg in 5 ml 'TRISMEG' buffer, pH 7.9). The supernatant, following centrifugation at 12000 *g* for 20 min, was applied to a column (2.5 × 13.2 cm) of DEAE-Sepharose (Pharmacia/P-L Biochemicals, Piscataway, NJ) established in 'TRISMEG' buffer, pH 7.9. The column was washed with 150 mM KCl in 'TRISMEG' buffer, pH 7.9 (120 ml), and the PAL activity was eluted with 250 mM KCl in 'TRISMEG' buffer, pH 7.9. The enzyme-containing fractions (14.6 ml) were transferred to a column (2.5 × 90.5 cm) of BioGel A1.5 (BioRad, Richmond, CA) established in 'TRISMEG' buffer, pH 7.9. The enzyme eluted in a vol. 1.25–1.40 times the void vol. Table 2 summarizes the stages of purification.

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