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Identification of an Aromatic Amino Acid Decarboxylase from a Yew-Associated Fungus

Abstract

Several fungi isolated from the inner bark of the Pacific yew tree (Taxus brevifolia) *have been studied as potential paclitaxel producers. Fungal isolate H10BA2, identified as* Penicillium raistrickii, *showed evidence of* de novo *paclitaxel production when grown in liquid culture. This fungus differed in several respects from isolates of* P. raistrickii *obtained from other sources, including the isolates available from ATCC (American Type Culture Collection). Soluble protein extracts of H10BA2 yielded a protein fraction that demonstrated aromatic amino acid decarboxylase activity, converting L-phenylalanine to phenethylamine. Isolation of the decarboxylase enzyme, identified for the first time in a fungus, as well as the characterization of the phenethylamine product is described.*

Key words: decarboxylase, *Penicillium raistrickii*, AADC, enzyme, amino acid decarboxylase.

Introduction

It has been proposed that aromatic amino acids are important precursors of secondary metabolites in higher plants, and that decarboxylation of these amino acids may be involved in regulatory mechanisms for the synthesis of these secondary metabolites (Kawalleck *et al.* 1993, Facchini and DeLuca 1994). Aromatic amino acid decarboxylases (AADC's) have been isolated from plant, insect, bacterial and animal sources, but have not been described from fungal sources (Marques and Brodelius 1988, Choudhury *et al.*1990, Tocher and Tocher 1972, Maneckjee and Baylin 1983). In animals, AADC's are important in the production of norepinephrine from tyrosine (Christenson *et al.* 1970) and the decarboxylation of L-Dopa and 5-hydroxytryptophan to form L-dopamine and serotonin, respectively (Maneckjee 1983, Albert 1987). In higher plants, secondary metabolite production can be correlated with various AADC's. In *Papaver somniferum*, L-Dopa decarboxylase is important in the biosynthesis of alkaloids, including morphine (Roberts and Antoun 1978) and in the production of 3-hydroxytyramine by *Cytisus scoparius* (Tocher and Tocher 1972). Specific L-tyrosine decarboxylases have been isolated from both barley roots and parsley (Kawalleck *et al.* 1993.)

This work identifies aromatic amino acid decarboxylase activity found in soluble protein extracts from cultures of *Penicillium raistrickii* that apparently

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catalyzes the conversion of L-phenylalanine to phenethylamine. An aromatic amino acid decarboxylase may be an important enzyme in the production of other secondary metabolites.

Material and Methods

General Experimental Procedures

Gas Chromatography/ Mass Spectrometer (GCMS) analyses were run on a Hewlett-Packard 5890 GC with a HP-5 capillary column and a HP5971 Mass Spectrometer. Thin layer chromatographies (TLC's) were run on Whatman AL SIL G/UV, 250 mm layer. Anion exchange resin was Whatman, 4057050 DE-52. All solvents were reagent grade. [$^2H_5$]-L-phenylalanine (five aromatic hydrogens were substituted with deuterium, ^2H) was purchased from Cambridge Isotope Laboratories, DLM-1258; and [U-^{14}C]-L-phenylalanine (universal labeled) was purchased from American Radiolabeled Chem. Inc., ARC 675. Fuji RX medical x-ray film was used for autoradiographs.

Fungal Fermentation

Isolates of H10BA2 (identified as *Penicillium raistrickii* by Dr. Zofia Lawrence at the International Mycological Institute) were grown in 5 L M1S medium (5 g Bacto-soytone, 60 g sucrose, 1 g yeast extract, per liter of broth). Both still cultures (20 day) and shaker cultures (6 day) were used for the preparation of soluble protein extracts. Cultures were filtered through Miracloth" (Calbiochem, 475855) and washed twice with 1 L volumes of 50 mM Tris-HCl (pH 7.6), 10 mM EDTA, 1 mM 2-mercaptoethanol, and 0.15 M NaCl (4° C). The mycelium was dried by squeezing in Miracloth" to remove excess moisture, placed in a pre-cooled mortar (-20° C), frozen with liquid nitrogen and ground to a fine powder with a pestle. This powder was resuspended in a 200 mL solution that was 50 mM Tris-HCl and 1 mM 2-mercaptoethanol (pH 7.6) and kept on ice. Cellular debris was removed by centrifugation at 22 800 *g* for 10 minutes. The supernatant, which contained the soluble protein fraction, was saved. This protein preparation was frozen in 1.5 mL aliquots (-20° C). A separate aliquot was saved for Bradford protein assay (Bradford 1976). Typical concentrations of protein as determined by the Bradford assay were in the range of 0.8 to 1.5 mg/mL. These protein preparations were used for enzyme assay procedures.

Enzyme Purification

Powdered $(NH_4)_2SO_4$ was added slowly to the crude supernatant at 4° C while stirring, to a final concentration of 1.0 M $(NH_4)_2SO_4$. The resulting slurry was centrifuged for 15 min at 22 800 *g*. The supernatant was applied to a column of Pharmacia phenyl-sepharose 6 fast flow (2.5 x 11.5 cm), previously equilibrated with HIC buffer 1, at a flow rate of 4 mL/min. The column was rinsed with 200 mL of HIC buffer 1. The enzyme was eluted by a stepwise gradient of HIC buffers [1.0 M, 0.8 M, 0.6 M, and 0.0 $(NH_4)_2SO_4$] using 200 mL of each solution, with 50 mL fractions collected. Five mL of each fraction was dialyzed against buffer C and assayed for enzyme activity by TLC analysis. Active fractions were combined and dialyzed against buffer C. A Pharmacia CM Sepharose Fast Flow cation exchange column (1.5 x 16.5 cm) was equilibrated with buffer C. Dialyzed fractions were loaded onto the column at 4 mL/min. The column was rinsed with 100 mL of buffer C. The enzyme was eluted by a stepwise gradient of CAT buffers (120 mM, 140 mM and 200 mM KCl) using 100 mL of buffer for each step, with collection of 10-mL fractions. Active fractions were dialyzed against buffer C and concentrated by column chromatography on a Pharmacia CM Sepharose Fast Flow

cation exchange column (1 x 6 cm). The enzyme was eluted by a stepwise gradient of CAT buffers (100 mM and 200 mM KCl), using 32 mL of buffer for each step, with collection of 4 mL fractions. Fractions were stored at -20°C.

SDS-PAGE analysis was performed according to the method of Laemmli 1970. Gels were made with a 4 percent acrylamide stacking gel and a 6 percent acrylamide separating gel, 1.5 mm thick. Reducing SDS-PAGE analysis was run in the presence of 262 mM 2-mercaptoethanol. Gels were stained by a silver stain method developed by Schoenle and Sammons 1984.

Buffers

We prepared buffers with reagent grade chemicals and adjusted pH with either HCl or NaOH. All chromatography buffers were based on a solution of 1 mM dithiothreitol, 1 mM pyridoxal-5-phosphate (Pxy-P), 50 mM sodium acetate, pH 5.0 (buffer C). *Hydrophobic interaction chromatography* (HIC) buffers 1, 0.8, 0.6, and 0 varied in the concentration of $(NH_4)_2SO_4$ (1.0 M, 0.8 M, 0.6 M and 0.0 M) added to buffer C respectively. Cation exchange chromatography (CAT) buffers varied in the concentration of KCl added to buffer C. CAT buffers 100, 120, 140 and 200 contained 100 mM, 120 mM, 140 mM and 200 mM KCl, respectively.

Enzyme Assay Protocol

We adapted the enzyme assay protocol from a method by Kurylo-Borowska and Abramsky 1972. Enzyme assay mixtures had a total volume of 1 mL. Each assay contained Tris-HCl buffer (pH 8.0, 50 mM), $MgCl_2$ (40 mM) and ATP (10 mM). L-phenylalanine was added to a final concentration of @ 4 mM. If [$^2H_5$]-L-phenylalanine were used, then it was added to a final concentration of 4 mM; if [U-^{14}C]-L-phenylalanine were used, then it was added to a specific activity of 3 µCi/mg (1 µCi/mL) with unlabeled L-phenylalanine. All ingredients except protein were mixed in 1.5 mL Eppendorf tubes on ice. The protein was added to give final concentrations of 0.25 to 0.5 mg/mL, and tubes were placed in a 30°C water bath. Incubation times up to 24 hours were used, but 30 minutes was sufficient to produce detectable product. At the end of the desired incubation time, the assay tubes were placed in boiling water for 1 minute to deactivate enzymes. The assay mixtures were then transferred to glass vials and dried *in vacuo*. Chloroform-methanol [0.5 mL of 1:1 (v/v)] was added to each vial after drying. This was mixed and allowed to incubate for 30 minutes at room temperature. The organic extract was analyzed by TLC for the presence of **1**.

Thin Layer Chromatography

Approximately 3 mL of the 1:1 chloroform-methanol solution from the enzyme assay was spotted on TLC plates. TLC plates were eluted with 1:1 chloroform-methanol. Plates were then air dried, sprayed with ninhydrin reagent (0.3 g ninhydrin in 100 mL *n*-butanol and 3 mL glacial acetic acid, Merck 1976) and warmed with a heat gun to identify the amine containing fractions. Phenethylamine gave a purple spot at R_f 0.86 under these conditions. L-Phenylalanine did not migrate from the origin.

Autoradiography

Enzyme assays using radiolabeled L-phenylalanine as substrate were applied to TLC plates, then analyzed by autoradiography. The plates were overlaid with the film in the dark and exposed for 2 to 6 weeks. Developed film was examined for spots that resulted from exposure to radioisotope.

Gas Chromatography-Mass Spectrometry

Analysis of enzyme assays by GC-MS for the presence of phenethylamine used the following GC parameters: injection port 280° C, column flow rate 0.5 mL/min.; oven temperature

program 130° C to 280° C (@ 10° C/ min.); MS interface 280° C. TIC's were recorded for natural and synthetic phenethylamine. SIM was used for [$^2H_5$]-phenethylamine due to low concentration of product.

Column Chromatography

DE-52 gel (25 g) was equilibrated with a buffer that was 0.05 M Tris-HCl (pH 7.6), 5 mM dithiothreitol, and 0.1 mM EDTA. The equilibrated gel was packed into a 2.4 x 10 cm glass column at 4° C using a persitaltic pump to maintain a constant flow rate of 1 mL/ min. Crude protein preparations were then loaded in a 100 mL volume of the above buffer system. The following stepwise elution system was used: buffer plus: 0.16 M KCl (2 x 10 mL); 0.21 M KCl (2 x 10 mL); 0.35 M KCl (2 x 10 mL); 0.45 M KCl (4 x 10 mL). Fractions were collected (10 mL) and assayed for protein concentration and phenethylamine production.

Isolation of Phenethylamine

Enzyme assay mixtures were dried *in vacuo* and extracted with 1 mL of chloroform-methanol (1:1, v/v). The volume was reduced *in vacuo* to 500 µL and the extract was applied to an HPLC silica gel column run in gradient mode from 10:1 chloroform-methanol to 1:1 chloroform-methanol.

Phenethylamine, 1

Physical characteristics : liquid, bp 196° C; MS m/z 121(14), 105(3), 91(100), 77(31), 65(88); ^{1}H NMR spectral data: (300 MHZ, CD_3OD) d 7.42-7.11 (5H, *m*), 3.18 (2H, *t*, *J*=7), 2.95 (2H, *t*, *J*=7).

Results and Discussion.

We examined the soluble protein extract of *Penicillium raistrickii* isolate H10BA2 for evidence of enzymes involved in paclitaxel biosynthesis. In particular, we were looking for a phenylalanine aminomutase, analogous to the enzyme isolated from the yew tree by Floss and his coworkers (Fleming *et al.* 1993, Walker and Floss 1998). In an attempt to define a parallel enzyme function in fungal production of paclitaxel, we performed enzyme assays on crude soluble protein extracts from H10BA2 using L-phenylalanine as a substrate. Several products were routinely detected in this assay. Product mixtures were applied to thin layer silica gel plates and eluted with chloroform-methanol (1:1, v/v). The mixtures resolved into discreet spots and were visualized with ninhydrin reagent. This assay consistently yielded compound **1** (R_f = 0.86) which generated a purple spot when sprayed with ninhydrin reagent. Enzyme preparations from six separate fungal fermentations, grown both still and as shaken cultures, all yielded protein extracts that produced compound **1** from phenylalanine.

When [U-^{14}C]-L-phenylalanine was used as a substrate, **1** was shown by autoradiography to have incorporated some radiolabel. The spot on the autoradiograms correlated perfectly to the ninhydrin-positive spot on the TLC plate (R_f = 0.86).

To isolate sufficient amounts of compound **1** to facilitate characterization, enzyme assay products were dried *in vacuo* and thoroughly extracted with chloroform-methanol (1:1). The organic extract was purified by silica gel HPLC. TLC analysis of each column fraction demonstrated the presence of **1**; this fraction was analyzed by GC-MS. Total Ion Chromatograph (TIC) exhibited a major peak at 8.1 min; mass spectral analysis of this peak showed a molecular ion at m/z 121, with major fragments at m/z 91 and 77 amu (Fig. 1).

When [$^2H_5$] -L-phenylalanine (five aromatic ring hydrogens substituted with deuterium) was used as a substrate for the enzyme assay, the TLC analyses were identical. [$^2H_5$]-compound **1** was again purified by HPLC and subsequently analyzed by GC-MS, this time using the more sensitive Selective

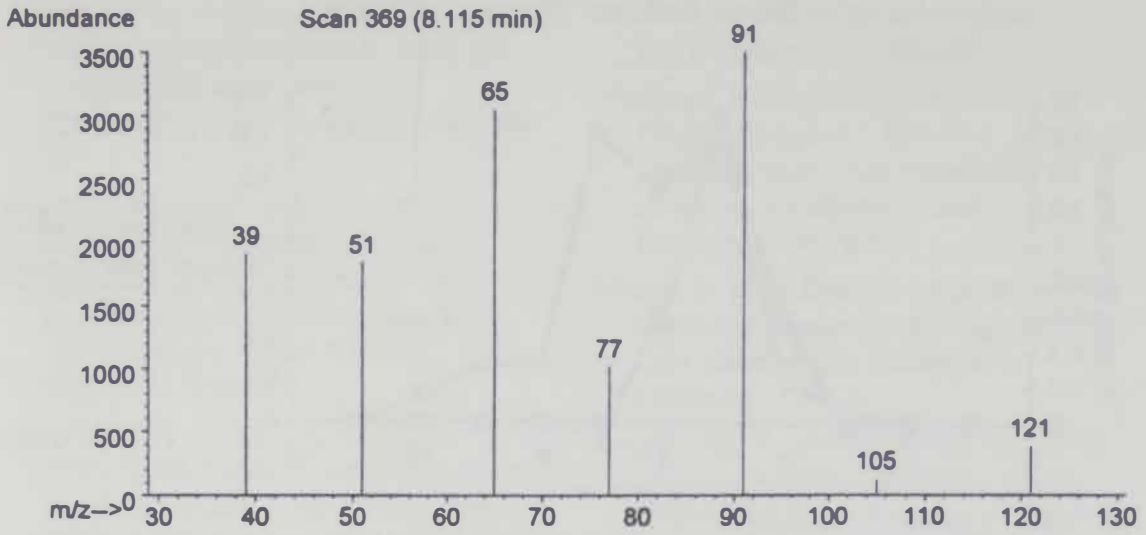

Figure 1. *Mass spectral analysis of the compound that correlated to the total ion chromatograph (TIC) peak at 8.1 min.*

Ion Monitoring (SIM) method instead of TIC. [$^2H_5$]-compound **1** eluted at 8.1 min; mass spectral analysis exhibited prominent peaks at m/z 126, 96 and 82 amu. These masses suggested the same fragmentation pattern as compound **1**, plus 5 amu from [$^2H_5$] incorporation.

Characterization of 1 was accomplished with combined mass spectral and NMR analyses. Reaction with ninhydrin indicated that it was a primary amine, and radiolabeling experiments indicated that it was an L-phenylalanine derivative. Proton NMR and mass spectral data of **1** were identical to that of authentic phenethylamine purchased from Aldrich to provide a TLC standard and to allow direct comparison of spectral data.

The enzyme-catalyzed conversion of L-phenylalanine to phenethylamine (Fig. 2) suggested the presence of an aromatic amino acid decarboxylase, previously unidentified in a fungus.

Attempts to purify the AADC began with anion-exchange column chromatography. Crude fungal protein extract (80 mg) was applied to a DE-52 column and eluted with increasing concentrations of KCl. Column fractions (10 mL) were collected and assayed for protein concentration by the Bradford procedure (Bradford 1976) and for AADC activity using the enzyme assay and TLC analysis as described above. Protein fractions 4, 5 and 6 catalyzed the transformation of L-phenylalanine to phenethylamine, **1**, in our enzyme assays. These fractions eluted after the application of 40 mL of solvent, which corresponded to 0.21 M KCl buffer (Fig.3).

A more rigorous isolation scheme confirmed the presence of an AADC in the fungal protein extract. Six-day shaker cultures were harvested as described above. Following ammonium sulfate precipitation and subsequent centrifugation, the supernatant was

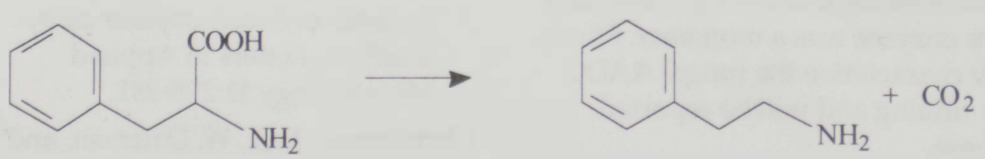

Figure 2. *Decarboxylation of L-phenylalanine to yield phenethylamine, **1**.*

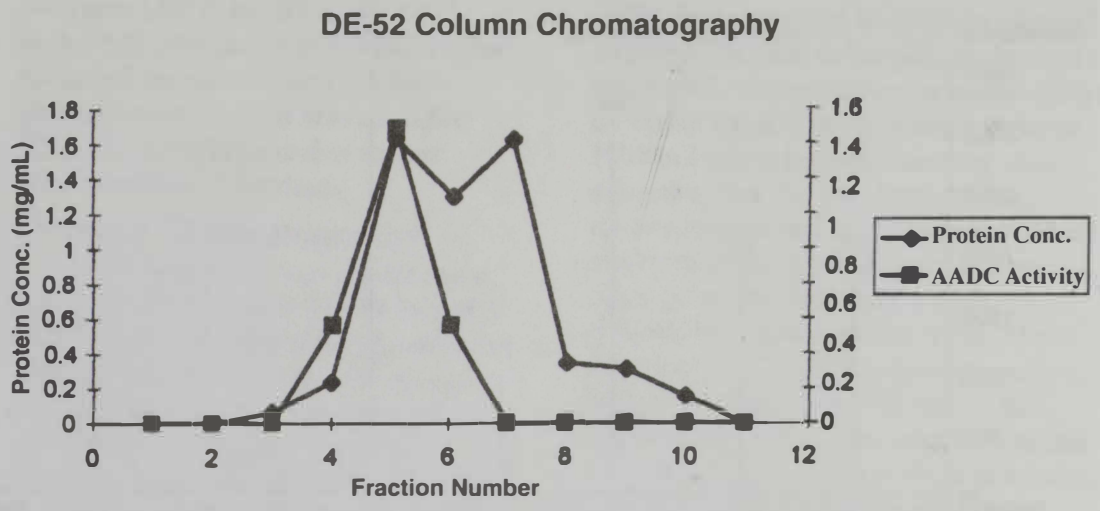

Figure 3. *Comparison of specific amino acid decarboxylase activity and protein concentration in anion exchange (DE-52) chromatography fractions of protein extract.*

applied to a phenyl-sepharose column equilibrated with hydrophobic interaction chromatography buffer 1 (HIC buffer). AADC activity was concentrated in the fractions that eluted with 0.6 M HIC buffer. The fractions were dialyzed and subjected to CM-Sepharose Fast Flow cation exchange chromatography. Active fractions yielded a single enzyme assay product with an R_f of 0.86. AADC activity was concentrated in the fractions that eluted with 140 mM KCl buffer. These active fractions were dialyzed and again applied to a CM-Sepharose Fast Flow cation exchange column, and eluted by a stepwise gradient of CAT buffers. Undialyzed fractions were tested for activity as described. A single enzyme product, **1**, was evident in the TLC assays of the AADC fractions, which eluted with the 200 mM KCl buffer.

The purity of the enzyme was demonstrated by SDS-PAGE analysis. Fungal AADC yielded a single band of 125 000 ± 3000 Da under both reducing and nonreducing conditions, indicating that the enzyme was a monomer. Efforts to fully characterize the fungal AADC are continuing and will be reported elsewhere.

Although AADC's are not involved in the biosynthesis of fungal taxol they may play a role in the formation of other fungal metabolites. The biosynthesis of the *Claviceps purpurea* metabolite ergotamine involves the decarboxylation of isoprenylated tryptophan (Floss 1976). The biosynthesis of b-carbolines by the fungus *Fusarium* sp. probably involves the decarboxylation of tryptophan.

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