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THE *CLAVICEPS PURPUREA* GENE ENCODING DIMETHYLALLYLTRYPTOPHAN SYNTHASE, THE COMMITTED STEP FOR ERGOT ALKALOID BIOSYNTHESIS+

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SUMMARY — The first pathway-specific step of ergot alkaloid biosynthesis in the fungus, *Claviceps purpurea*, is catalyzed by the prenyltransferase, 4-(γ,γ-dimethylallyl)tryptophan synthase. Partial sequence information was obtained for the purified enzyme and a degenerate oligonucleotide mixture was used to identify and amplify segments of the gene, *dmaW*. The complete gene and near-full-length cDNA were cloned and sequenced. The cDNA was cloned in a yeast expression vector in sense and antisense orientations relative to the inducible *GAL1* promoter. Extracts of yeast transformants with the sense constructs, but not antisense constructs or cloning vector, catalyzed production of 4-(γ,γ-dimethylallyl)tryptophan. The sequence of *dmaW* and its cDNA indicated that it encoded a 455 amino acid polypeptide with a predicted molecular mass of 51,824 Da and a putative prenyl diphosphate binding motif.

Ergot alkaloids, produced by plant parasitic fungi such as *Claviceps purpurea*, are neurotropic toxins and important pharmaceuticals (1, 2). Synthesis of DMAT, catalyzed by dimethylallyl diphosphate:L-tryptophan dimethylallyltransferase (dimethylallyltryptophan synthase), is the first pathway-specific and probably a rate-limiting step in ergot alkaloid biosynthesis (3, 4). The enzyme has been purified from *C. purpurea* ATCC 26245, and its catalytic mechanism described (5, 6). Gel filtration and electrophoretic analysis suggest the enzyme is a homodimer with 52-kDa subunits (5). Here we report the cloning of the dimethylallyltryptophan synthase gene, *dmaW*, and its cDNA from *C. purpurea*, and we confirm the identity of *dmaW* cDNA by expression in transformed yeast.

+*Nucleotide sequence accession number*: L39640.

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ABBREVIATIONS — *C.*, *Claviceps;* DMAPP, dimethylallyl diphosphate; DMAT, 4-(γ,γ-dimethylallyl)tryptophan; *dmaW*, dimethylallyltryptophan synthase gene; I, inosine nucleoside; kb, kilobasepair; X, unknown amino acid; Y, pyrimidine nucleoside.

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MATERIALS AND METHODS

Biological Materials and Growth Media

Claviceps purpurea ATCC 26245, from the American Type Culture Collection, was grown on potato dextrose agar (Difco Laboratories, Detroit, MI) for DNA isolation. For RNA isolation, *C. purpurea* was grown in NL-406 medium without sucrose and harvested during rapid ergot alkaloid accumulation (5). *Saccharomyces cerevisiae* (yeast) EY957, from E.A. Elion (Harvard Medical School, Cambridge, MA), was grown in YP medium (1% Bacto-yeast extract, 2% Bacto-peptone [Difco]) (7) amended with 2% sucrose or Gal as indicated, or with 2% Glc for routine culture.

General Protocols

Materials and procedures for nucleic acid isolation, analysis, cloning, sequencing, and yeast transformation are described elsewhere (7-11).

Protein Fragmentation for Sequencing

Purified dimethylallyltryptophan synthase (2 mg) (5) was treated with CNBr (12) (CAUTION, use fume hood), and the resulting peptide fragments were fractionated by microbore HPLC with a Michron BioResources Ultrafast Microprotein Analyzer using a PLRP-S 300A (Polymer Laboratories, Amherst, MA) 1 × 50 mm column. The mobile phase was a linear gradient of 0.1% $F_3CCO_2H_{(aq)}$ to acetonitrile. Fractions containing peptide were collected and sequenced by Edman degradation (12) using a Perkin-Elmer, Applied Biosystems Div. (Foster City, CA) model 477A peptide sequencer.

Southern-Blot Hybridization Analysis

Southern-blot hybridization with radiolabelled oligonucleotides was based on published methods (7, 9). Oligonucleotide mixture 4018 (TAIACYTGIGGYTCIGGCAT, where I = inosine and Y = C or T, complementary to *dmaW* codons 357-351 in Fig. 1) was 5'-labelled using T4 polynucleotide kinase and [γ-^{32}P]ATP. Membranes were prehybridized overnight at 37 °C in 0.9 M NaCl, 0.9M Na_3citrate (6 × SSC), 5 × Denhardt's solution (7), 0.05% Na_4*PP*i, 0.5% Na_2dodecyl-SO_4 and 100 μg ml^{-1} denatured herring sperm DNA. The solution was replaced with 3 M Me_4NCl, 10 mM sodium phosphate pH 6.8, 1 mM EDTA, 0.5% Na_2dodecyl-SO_4, 100 μg ml^{-1} denatured herring sperm DNA, 0.1% non-fat dried milk (Carnation Co., Los Angeles, CA), and the ^{32}P-labelled oligonucleotide mixture. The membranes were incubated 46 h at 49 °C, rinsed at room temperature with 3 M Me_4NCl, 50 mM Tris-HCl pH 8.0, 0.2% Na_2dodecyl-SO_4, incubated 1 h with the same solution at 49 °C, gently shaken with 2 × SSC, 0.1% Na_2dodecyl-SO_4 for 10 min at 49 °C, then autoradiographed.

Plasmid Vectors and Oligonucleotide Primers

T-vector forms of pBCKS+ and pBluescriptKS+ (Stratagene Cloning Systems, La Jolla, CA) were prepared by *Eco*RV digestion followed by addition of dT to the 3'-ends, using *Taq* DNA polymerase (Perkin-Elmer). The yeast expression vector, pMDM281, was generated from pMDM152 (13), and contained unique *Bam*HI and *Nde*I sites between the yeast *GAL1* gene promoter and the 3' portion of *SIC1*. Oligodeoxyribonucleotide primers for PCR included: linker primer (5'-$[GA]_{10}$ACTAGTCTCGAGT_{18}-3'); and primer numbers 1305 (5'–C_{13}–3'); 2745 (CGGAATTAACCCTCACTAAAG, flanking the *Kpn*I site of pBCKS+); 4018 (described above); 7442 (CGGTGGAAGATCTGTGGACGTTGG, *dmaW* codons 284-290); 7529 (ACCCAGAACAAGCTAGGCGT, *dmaW* codons 170-176, with an error); 7530 (AACGTCCACAGATCTTCCAT, complementary to *dmaW* codons 290-284); 7597 (GCGTCGACGCAAAGACCCTTGACAT, with a *Sal*I site and complementarity to the *dmaW* cDNA 3'-end); 7801 (CAAGTAACCGAGAAAATGATGACAAAAGCTCCAG, with 3'-end homology to *dmaW* codons 1-7); and 7948 (GCGTCGACACGATGATGACAAAAGC, with a *Sal*I site and the 5'-end of the *dmaW* coding sequence).

PCR Amplification and Cloning of Genomic DNA and cDNA

All PCR were in 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, and 200 μM each dNTP, with primers (each at 120 pmol ml^{-1} or as indicated), specified template, and 25 units ml^{-1} cloned *Taq* DNA polymerase (Perkin-Elmer). Temperature regimes were 94 °C for 3 min, temperature cycles as specified, 72 °C for 5 min, and 4 °C.

A genomic DNA segment was amplified using template prepared by ligating *Hin*dIII-digested *C. purpurea* DNA with *Hin*dIII-digested pBCKS+ in a 1:10 (w/w) mixture. A 100 μl reaction contained *ca.* 30 ng template DNA. Initially, 50 pmol primer 4018 was added, and PCR involved five cycles of 94 °C for 45 s, 61 °C for 45 s, and 72 °C for 2 min; then 12 pmol vector

primer 2745 was added and PCR was continued for 35 cycles. The product was cloned into pBCKS+ T-vector to produce pKAES130.

Each amplification of cDNA was by two successive rounds of PCR. Template for the first round was cDNA synthesized by reverse transcription (7) using Superscript II (Gibco BRL, Gaithersburg, MD) and either linker primer or, for the 5'-end, the degenerate primer mixture 4018. Four µl of first-round product was template for each 100 µl second-round PCR. The cDNA 3'-end was amplified using, for both rounds, primer 7442 and linker primer, and 38 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 90 s. The cDNA 5'-end was synthesized by reverse transcription with primer 4018, tailed using terminal deoxynucleotidyltransferase (Gibco BRL) and dGTP, and amplified in two rounds: first with primers 4018 and 1305 and 40 cycles of 94 °C for 30 s, 55 °C annealing for 30 s, and 72 °C for 1 min; then with primers 7530 and 1305 and the same temperature cycles except that annealing was at 58 °C. Near-full-length *dmaW* cDNA was amplified in two rounds using: linker primer and primer 7801 with 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 150 s; then primers 7801 and 7597 with 31 similar cycles. The product was ligated into pBluescriptKS+ T-vector to produce pKAES132.

Construction and Screening of Genomic Clone Library

Size-selected *C. purpurea* DNA fragments from incomplete *Mbo*I digestion were cloned in cosmid pMOcosX (14). Each of 3,840 *Escherichia coli* XL1-blue transfectant colonies was inoculated into 0.75 × LB broth (7) with 25% glycerol and 100 µg ml^{-1} ampicillin in a well of a 24-well microtitre plate, grown overnight with gentle shaking, then transferred to a well of a 96-well microtitre plate. Plates were replicated using a Boekel pin replicator (Curtin Matheson Scientific, Florence, KY), and stored at -80 °C. A pool of all clones from each plate was inoculated into 5 ml LB with ampicillin, and shaken overnight at 30 °C. Cells were pelleted and lysed 15 min at 95 °C in 1% Triton X-100, 20 mM Tris-HCl pH 8.5, 2 mM EDTA. Samples (1 µl) were added to 20 µl mixtures for PCR using primers 7529 and 7530, and 35 cycles of 94 °C for 30 s, 61 °C for 30 s, and 72 °C for 45 s. Each row of the positive plate, then clones of the positive row, were screened to identify cosmid clone, pCP26-11E.

Gene Expression in Yeast

The cDNA clone pKAES132 served as template for PCR using primers 7948 and 7597. The product was digested with *Sal*I, half-filled in using DNA polymerase large fragment, dCTP and dTTP (7), and cloned into the half-filled (with dG and dA) *Bam*HI site of pMDM281. Restriction endonuclease site mapping indicated the orientation of *dmaW* coding sequence relative to the *GAL1* promoter in each clone. Yeast cells were transformed by the Li-acetate method (7) and plated for selection on CM dropout agar without leucine (7). Transformant cells were grown at 30 °C in selective CM broth to late log phase, then 60 ml YP (7) with 2% sucrose was inoculated to an initial A_{600} of 0.08. The culture was grown to mid-log phase (A_{600} 1-2) *ca.* 10 h, 30 °C with shaking at 230 rpm. The cells were pelleted by centrifugation (7,600 × *g*, 5 min), washed with sterile water, resuspended in 160 ml YP with 2% Gal, shaken for 12 h at 30 °C, then harvested by centrifugation. The cell pellet (100-200 µl in a 1.7 ml microcentrifuge tube on ice) was resuspended in one volume cold extraction buffer (5), and two volumes cold, acid-washed glass beads (425-600 µm) were added. The cells were disrupted by seven cycles each of vortexing at maximum speed for 45 s followed by 60 s on ice. The extract was removed by pipetting, the glass beads were washed with one volume cold extraction buffer, and the wash was combined with the extract. Cells and debris were pelleted by 1 h centrifugation at 12,000 × *g*, 4 °C, and the cell-free extract was collected by pipetting and assayed for enzyme activity as described below.

Enzyme Assays and Authentication of DMAT

L-Trp-dependent incorporation of the radiolabelled prenyl moiety of [1-^{3}H]DMAPP was assayed by the method of Krupinski *et al.* (3) with modifications. To prepare cell-free extract from *C. purpurea*, lyophilized mycelia (*ca.* 10 mg) in 0.3 ml cold extraction buffer with freshly added 1 mM phenylmethylsulfonylfluoride, was sonicated on ice 3-5 times, 7 s each, with a sonic disrupter (model TM-50, Tekmar, Cincinnati, OH) at setting 75. The extract was incubated 30 min on ice, then centrifuged 17,000 × *g* for 20 min at 4 °C. The supernatant was assayed directly or stored at -80 °C. Yeast cell-free extracts were prepared as described above. Enzyme assay mixtures contained 4 µl of 5 × assay buffer (50 mM Tris-HCl pH 8.0, 100 mM 2-mercaptoethanol, 100 mM $CaCl_2$, 50% glycerol), 0.2 µl of 10 mM DMAPP, 0.05 µCi [1-^{3}H]DMAPP (15 Ci $mmol^{-1}$; American Radiolabelled Chemicals Inc., St. Louis, MO), 2 µl of 10 mM L-Trp, 6 µl of cell-free extract and water to 20 µl. Reactions were run for 15 min at 30 °C, then stopped by addition of 4 µl of 2 N HCl in 80% ethanol. The mixtures were incubated 20 min at 30 °C to hydrolyze unreacted DMAPP, then 150 µl H_2O was added to each, and the open tubes were placed in sand in

wells of a 100-110 °C heat block for 90 min to remove radioactive volatiles (CAUTION, use fume hood). The contents of each tube were redissolved in 150 µl H_2O and transferred into a scintillation vial with 4 ml scintillation fluid, and radioactivity was determined by liquid scintillation spectrometry. Background incorporation was determined by reactions without L-Trp. Protein concentrations were measured by Bradford dye-binding (7).

The products from incubation of cell-free extracts with L-Trp and DMAPP were analyzed by a modification of the procedure of Gebler *et al.* (6). Each 1 ml reaction mixture contained 300 µl yeast cell-free extract, 50 mM Tris-HCl pH 7.8, 10% glycerol (v/v), 500 µM L-Trp, and 1 mM DMAPP or [^{3}H]DMAPP. The mixture was incubated 24 h at 30 °C. Proteins were removed by filtration through an Amicon (Beverly, MA) Microcon-10 concentrator, and the filtrate was lyophilized. The residue was dissolved in 0.1% $F_3CCO_2H_{(aq)}$ and 10% acetonitrile, applied to a Microsorb MV C18 (Rainin Instrument Co., Woburn, MA) reversed-phase HPLC column, and eluted with a 30 ml linear gradient of 1:10 to 6:4 (v/v) acetonitrile/0.1% $F_3CCO_2H_{(aq)}$. For analysis of unlabelled products, elution was monitored simultaneously at 214 nm, 269 nm, and 280 nm, and uv spectra obtained for each peak using a photodiode array detector. The peak with the same retention time as authentic DMAT (5) was collected and lyophilized, then dissolved in 0.1% $F_3CCO_2H_{(aq)}$ and analyzed by positive ion fast atom bombardment MS. For analysis of labeled product, the elution from HPLC was monitored at 214 nm, and radioactivity of fractions was measured by liquid scintillation spectrometry.

RESULTS AND DISCUSSION

Inferred amino acid sequences of three CNBr-generated peptide fragments of purified *C. purpurea* dimethylallyltryptophan synthase were Met-Ala-Lys-Lys-Tyr-Arg-Val-Phe-Leu-Glu-Gly-Ser-Phe-Pro-X-X-Asp-Phe-Glu-Ser-Leu-X-Tyr-Leu (fragment 1), Met-Val-His-Tyr-Ala-Leu-His-Pro-Asp-Gln (fragment 2), and Met-Pro-Glu-Pro-Gln-Val-Tyr-Phe-Thr-Val (fragment 3). The initial methionines were inferred because CNBr cleaves only on the *C*-terminal side of Met, and the *N*-terminus of the uncleaved protein was blocked to Edman degradation. Primer 4018, a degenerate oligonucleotide mixture encoding part of fragment 3, was labelled and hybridized to a Southern blot of *C. purpurea* genomic DNA. The probe hybridized to two *Hin*dIII fragments (2.65 kb and 7.6 kb), and two *Xho*I fragments (3.6 kb and 7.6 kb). A 692 basepair segment of the smaller *Hin*dIII fragment was amplified, cloned, and sequenced. An open reading frame extended the length of the DNA segment, was in the expected frame with primer 4018, and also encoded peptide fragment 2. The DNA segment hybridized to a 2.65 kb *Hin*dIII fragment and a 7.6 kb *Xho*I fragment of *C. purpurea* DNA, and to no additional DNA fragments (data not shown).

In total, 3,840 cosmid clones of *C. purpurea* genomic DNA were screened by PCR, and one putative *dmaW* clone, pCP26-11E, was identified. Southern-blot analysis of this cosmid identified a 3.3 kb *Eco*RI fragment that extended from a genomic *Eco*RI site 2.3 kb upstream of the *Xho*I site in the *dmaW* coding region (see Fig. 1), downstream to the vector site flanking the genomic DNA insert (data not shown). This *Eco*RI fragment was subcloned in pBCKS+ to produce pKAES131. The *dmaW* sequence determined from this clone is shown in Figure 1.

The 5' and 3' portions of cDNA were amplified, cloned, and sequenced. The first ATG in the 5'-end was assumed to be the translational start site and, therefore, has been designated codon one (Fig. 1). Comparison of nucleotide sequences of the cDNA clones with the genomic sequence indicated three transitional differences (data not shown). A near-full-length cDNA was also amplified, cloned to produce pKAES132, and sequenced. This cDNA had three other transitions (positions 378, 846 and 1522 in Fig. 1), none of which changed the inferred protein sequence. Although sequences of the cDNA clones were not identical, each nucleotide position of the genomic sequence in Figure 1 was verified in at least one cDNA. There were two introns near the

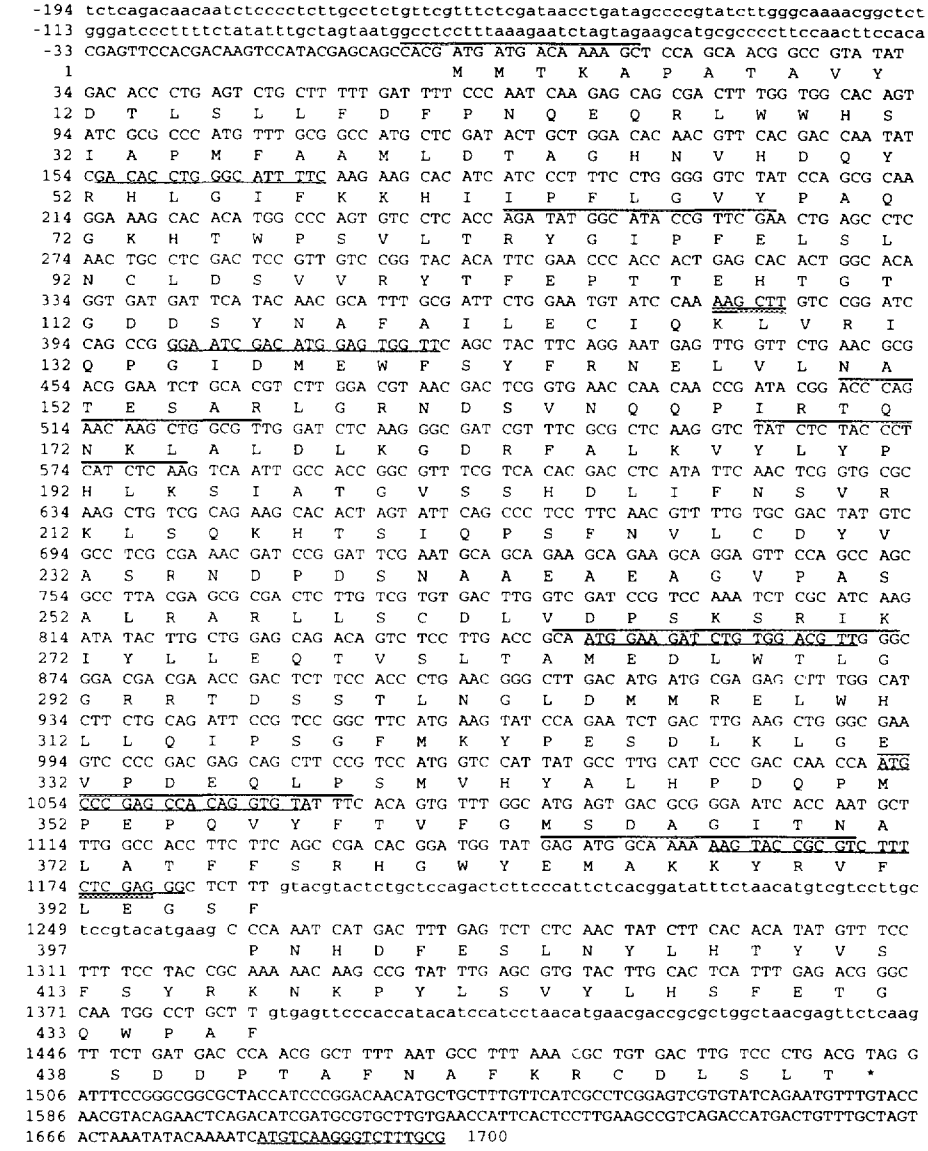

Figure 1. Sequence of *Claviceps purpurea dmaW* encoding dimethylallyltryptophan synthase. Sequences in uppercase were also in cloned cDNA of *dmaW* mRNA. The predicted polypeptide sequence is listed beneath the DNA sequence. Numbers at left refer to nucleotide sequence and codon positions, whereby the A of the first ATG codon in the cDNA is designated position one and numbering proceeds 5' to 3'. Double-underlines indicate cleavage sites for *Hin*dIII (AAGCTT) and *Xho*I (CTCGAG). DNA sequences of sense primers are indicated by overbars, and those complementary to antisense primers are underlined.

3'-end of the coding sequence, and an amber stop codon followed codon 455. Assuming the universal genetic code, *dmaW* encoded a 51,824 Da polypeptide.

To confirm that *dmaW* encoded dimethylallyltryptophan synthase, the entire coding sequence was amplified from cDNA clone pKAES132, and subcloned into a yeast expression vector. The *dmaW* reading frame was in the sense orientation relative to the adjacent *GAL1*

promoter in subclones pKAES135 and pKAES136, and in the antisense orientation in subclones pKAES137 and pKAES138. Yeast transformed with each subclone or the vector was grown and induced with Gal, then cell-free extract was assayed for activity catalyzing L-Trp-dependent incorporation of label from [^{3}H]DMAPP. Extracts from yeast with pKAES135 and pKAES136 had specific activities (mean ± SE) of 171 ± 1.2 and 240 ± 6.7 pmol min^{-1} mg^{-1}, respectively. In contrast, specific activities measured in antisense and vector controls were 1-13 pmol min^{-1} mg^{-1}. The specific activity from *C. purpurea* extract was 591 ± 20.8 pmol min^{-1} mg^{-1}. After prolonged reactions with extract from yeast with pKAES135, the product was purified by HPLC. Material eluted with the same retention time (between 17.4 and 18.1 min) as that of authentic DMAT (5), and was not detected in antisense and vector control experiments (Fig. 2). The uv spectrum of this material had a maximum absorbance at 269 nm and was identical to that of authentic DMAT. Furthermore, fast atom bombardment MS of this material had a peak at m/z 273 (M+1), as expected for DMAT (5). Products of incubations with [^{3}H]DMAPP and L-Trp were also separated by HPLC, and a radioactive peak comigrating with authentic DMAT was obtained in experiments with the pKAES135 transformant, but not with the antisense or vector control. Thus, only extracts from yeast transformants with cDNA in the sense orientation directed synthesis of DMAT, demonstrating that the cloned gene encoded dimethylallyltryptophan synthase.

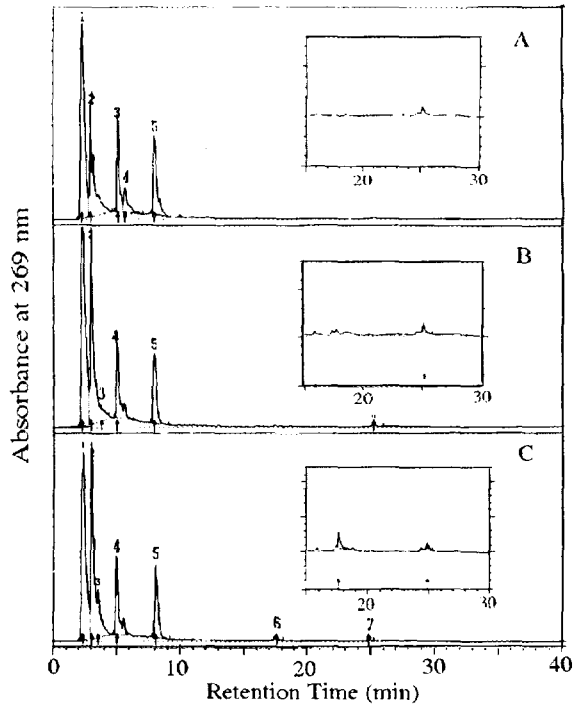

Figure 2. HPLC profiles of products from incubation of DMAPP, L-Trp, and cell-free extracts from Gal-induced cultures of yeast transformed with pMDM281 (Panel A), pKAES137 (Panel B), and pKAES135 (Panel C). Inserts are expansions of the 15-30 min regions of the elution profiles. Peak 6 in panel C was identified as DMAT by uv spectroscopy and MS.

The inferred sequence of dimethylallyltryptophan synthase included a possible prenyl diphosphate binding motif, Asp-Asp-Ser-Tyr-Asn, located at amino acid positions 113-117 (Fig. 1). Farnesyl diphosphate synthase from yeast has two Asp-Asp-X-X-Asp motifs which are involved in substrate binding, and are conserved in other farnesyl diphosphate and geranylgeranyl diphosphate synthases (15). This motif and a nearby Lys (codon 127) were the only similarities identified in computer comparisons of dimethylallyltryptophan synthase and other prenyltransferase sequences. The cloned dimethylallyltryptophan synthase gene will facilitate investigating the role of the enzyme in regulating ergot alkaloid production.

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