Overproduction of Soluble Trichodiene Synthase from
Fusarium sporotrichioides in Escherichia coli

David E. Cane,* Zhen Wu,* John S. Oliver,* and Thomas M. Hohn†

*Department of Chemistry, Box H, Brown University, Providence, Rhode Island 02912; and †Mycotoxin Research Unit,
USDA/ARS, National Center for Agricultural Utilization Research, Peoria, Illinois 61604

Received August 10, 1992, and in revised form September 18, 1992

Trichodiene synthase is a sesquiterpene cyclase isolated from various fungal species which catalyzes the cyclization of farnesyl diphosphate (FPP) to trichodiene. The trichodiene synthase gene (Tox5) of Fusarium sporotrichioides has previously been cloned and expressed as 0.05–0.1% of total cell protein in Escherichia coli. We have used polymerase chain reaction to amplify the trichodiene coding sequence carried on the plasmid pTS56-1. The resulting DNA, carrying a BamHI restriction site and the T7 gene 10 ribosome binding site and translational spacer element immediately upstream of the ATG start codon as well as a HindIII site adjacent to the translational stop codon, was inserted into the corresponding sites of the expression vector pLM1. The latter vector carried the promoter and translational leader sequence from T7 gene 10 and the E. coli rmBT₂T₂ tandem transcription terminator. This construct was cloned into E. coli BL21(DE3). The resulting transformants, when induced with isopropyl β-D-thiogalactoside, produced trichodiene synthase as 20–30% of total soluble protein. The recombinant synthase, which could be purified five-fold to homogeneity by ammonium sulfate precipitation, ion-exchange chromatography on Q Sepharose, and gel filtration on Superose 12, was identical to native protein in steady-state kinetic parameters and mobility on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and had the expected MENFP N-terminal sequence.

Sesquiterpene synthases catalyze the cyclization of the universal acyclic precursor farnesyl diphosphate (FPP) to any of 200 known cyclic sesquiterpenes. Although only a relative handful of these enzymes have been isolated and subjected to mechanistic study, they are all operationally soluble proteins, either monomers or homodimers of subunit \( M_r \), 40,000–60,000, and require no cofactor other than a divalent metal cation, Mg²⁺ usually being preferred (1–3). By far the best studied of these cyclases has been trichodiene synthase, a fungal protein responsible for the conversion of FPP to trichodiene (3), the parent hydrocarbon of the trichothecane family of mycotoxins (4, 5). Extensive mechanistic studies have supported a cyclization mechanism in which FPP is initially rearranged to its tertiary allylic isomer, nerolidyl diphosphate (NPP, (2)), which in turn undergoes further ionization, cyclization, and rearrangement to give trichodiene (6–9) (Scheme I). All the various electrophilic reactions and rearrangements are believed to take place at a single active site. According to this picture, the folding of the acyclic substrate at the active site is a major determinant of the structure and stereochemistry of the eventually formed sesquiterpene product (10).

Trichodiene synthase, which was first isolated from apple mold fungus Trichothecium roseum (11), has been purified to homogeneity from the T-2 toxin producer, Fusarium sporotrichioides and has been shown to be a homodimer of 45-kDa subunits (12). The closely related trichodiene synthase of Gibberella pulicaris (anamorph F. sambucinum) has been partially purified as well (13). Screening of a λgt11 genomic library of F. sporotrichioides DNA with antibodies to the cyclase resulted in isolation of the structural gene for trichodiene synthase (Tox5), encoded in a 1182-bp open reading frame containing a 60-nt in-frame intron (14). In vitro excision of the intron and subcloning of the coding sequence into the Escherichia coli expression vector gave a recombinant plasmid (pTS56-1) which could be used to express the enzyme in E. coli. The resulting transformants, when induced with isopropyl β-D-thiogalactopyranoside, produced trichodiene synthase as 20–30% of total soluble protein. The recombinant synthase, which could be purified five-fold to homogeneity by ammonium sulfate precipitation, ion-exchange chromatography on Q Sepharose, and gel filtration on Superose 12, was identical to native protein in steady-state kinetic parameters and mobility on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and had the expected MENFP N-terminal sequence.


1 This investigation was supported by a grant from the National Institutes of Health, GM30301, to D.E.C.
2 To whom correspondence should be addressed.
3 Abbreviations used: FPP, farnesyl diphosphate; NPP, nerolidyl diphosphate; PCR, polymerase chain reaction; EDTA, ethylenediaminetetra-acetic acid disodium salt; LB, Luria–Bertani; IPTG, isopropyl β-D-thiogalactopyranoside; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; rbs, ribosome binding site.
**OVERPRODUCTION OF TRICHODIENE SYNTHASE IN _Escherichia coli_**

The _Escherichia coli_ expression vector pDR540 resulted in bacterial expression of trichodiene synthase, albeit at relatively low levels corresponding to 0.05–0.1% of total cellular protein (15). Interestingly, transformed cells produced trichodiene itself at levels of up to 60 μg/liter, presumably due to utilization of endogenous FPP by the recombinant cyclase. The _F. sporotrichioides_ Tox5 gene has also been cloned into tobacco (_Nicotiana tabacum_), resulting in heterologous expression of trichodiene synthase and the production of detectable quantities of trichodiene in the plant host (16).

The corresponding trichodiene synthase gene of _G. pulicaris_ has also been isolated by using the _F. sporotrichioides_ gene as a hybridization probe to screen a library of _G. pulicaris_ DNA (17). The two sequences showed an 89% homology at the nucleotide level, including the 60-nt intron, and a 96% identity at the amino acid level, the primary difference lying in the presence of an additional nine amino acids at the C-terminus of the _G. pulicaris_ synthase. Although trichodiene synthase had no overall homology to any other known gene, significantly, both synthases were found to contain an aspartate rich motif previously noted in several isoprenoid chain elongation enzymes, including FPP synthase (18). Thus the trichodiene synthase of _F. sporotrichioides_ had the sequence VLDDSKD starting at amino acid 98, while the _G. pulicaris_ cyclase had the sequence VLDDSSD at the same position. Furthermore, both enzymes contained a short sequence rich in basic amino acids (DHRYR—_F. sporotrichioides_, DHRYR—_G. pulicaris_) closely analogous to a presumptive active site peptide previously implicated in pyrophosphate binding by avian FPP synthase (19). Nothing more is known currently about the active site of trichodiene synthase, or indeed any other sesquiterpene synthase. We therefore sought to obtain sufficient quantities of enzyme for further mechanistic and enzymological studies. We now report the high-level overexpression of trichodiene synthase and the purification and preliminary characterization of the recombinant cyclase.

**EXPERIMENTAL PROCEDURES**

**Materials.** Plasmid pLM1 and _E. coli_ strain BL21(DE3) were gifts from Professor Gregory L. Verdine of Harvard University. _E. coli_ strain XLI-Blue was from Stratagene (San Diego, CA) and strain JM109(DE3) was from Promega (Madison, WI). Restriction enzymes _SacI_ and _BamHI_ were from Stratagene and _HindIII_ was from T4 DNA ligase was purchased from Promega. _Pfu_ DNA polymerase was obtained from Stratagene. Oligonucleotides were synthesized by the phosphoramidite method on a BioSearch 8700 by Charles Sutterland. Bulk matrix Q Sepharose and Superose 12 were obtained from Sigma (St. Louis, MO) and Bio-Gel P-6DG was purchased from Bio-Rad (Richmond, CA). [1-3H]FPP (16.7 μCi/μmol) was synthesized as previously described (7).

All other materials used for recombinant DNA manipulations, enzyme assay, and protein purification were analytical grade or higher. All buffers and nutrient broths were prepared with doubly deionized nanopure grade water.

**Methods.** Restriction endonuclease digestions, DNA ligations, preparation and transformation of competent cells, plasmid minipreps, and other standard recombinant DNA manipulations were carried out according to published procedures (20), except that after restriction enzyme digestion, a Millipore Ultrafree-Probind filter unit was used to remove protein in place of phenol-chloroform extraction. Maxipreps of plasmid DNA by the alkaline lysis method (20) followed by precipitation with 13% polyethylene glycol were used to prepare double-stranded DNA for sequencing. DNA sequencing on plasmid products was carried out by the Sanger dideoxy chain termination method using the Sequenase 2.0 kit (U.S. Biochemical) according to the standard protocols and with primers developed earlier for the sequencing of the trichodiene synthase gene (14). PCR was carried out in a Coy thermal cycler (Ann Arbor, MI). SDS-PAGE was performed in 14 cm × 14 cm × 1 mm 12% gels by the method of Laemmli (21) and proteins were visualized by staining with Coomassie brilliant blue R-250. Densitometric analysis of dried SDS-PAGE gels was performed on a LKB Ultrascan XL laser densi-
tometer. Immunoblot detection of trichodiene synthase was carried out as previously described using Immobilon-P PVDF transfer membranes (Millipore) and rabbit antisera against F. sporotrichioides trichodiene synthase (14). Radioactivity measurements were obtained using 10-ml solutions of Optifluor (Packard) in a Beckman LS8010 liquid scintillation counter.

Construction of the pLM1-50 overproducer by PCR. Plasmid pTS56-1, previously described as pTS46-10A (470–529) and containing the F. sporotrichioides trichodiene synthase coding sequence in pDRS40 (15), was linearized by digestion with ScaI and the resulting DNA was used as template for the PCR method of Macferrin et al. (22). The start primer (Primer 1) was a 56-mer oligonucleotide containing a BamHI restriction site (underlined), the T7 gene 10 ribosome binding site (rbs) and translational spacer element (italics), and the first nine codons of the trichodiene synthase gene, including the start codon (bold) [5'-dTATTCGCCGATCCAGGATATCATGACAGGAGTACCTCCGACTCAG-3']. The reverse or hPrimer (Primer 2) was a 43-mer containing a HindIII restriction site (underlined) and the complementary sequence corresponding to the stop codon (bold) and the last eight codons of trichodiene synthase [5'-dCCGACGTTTCCATCGTACATATGGGAGTTTTACCTCCGACTCAG-3']. A typical 100-ml reaction was made up to contain sterile H2O (50 ml), 100 mM MgCl2 (10 ml), 10X buffer (1 ml) consisting of 100 mM Tris, pH 8.2, 100 mM KCl, 60 mM (NH4)2SO4, 20 mM MgCl2, 1% Triton X-100, and 100 ng/ml nuclease-free BSA), mixture of four dNTPs, each at 10 mM (2.5 ml), Primer 1 (20 pmol), Primer 2 (20 pmol), linearized pTS56-1 (0.5 ng), and pfu polymerase (1 ml, 2.5 units). All reactants except the polymerase were added in the order given to a screw cap 0.5-ml Eppendorf tube which was placed in the thermal cycler before starting the following program: (a) 96°C, 5 min; 65°C, 3 min; 72°C, 2 min (extension); (b) 96°C, 5 min; 65°C, 3 min (extension); (c) 78°C, 2 min (denaturation); (d) 78°C, 5 min (annealing); (e) 78°C, 2 min (extension); (f) 4°C, hold. The reactants were next heated to 96°C for 5 min then moved during the 3-min 65°C step, chilled rapidly in an ice bath before addition of primerase and mineral oil, briefly centrifuged, and replaced in the cycler at 65°C. The typical yield was 2–5 μg of amplified DNA per 100 μl reaction mixture. The PCR product was analyzed on a 1.5% agarose gel and purified using Millipore Pro-Bind and Ultrafree-MC 30,000 MW filter sets. The recovered DNA was digested sequentially with BsmHI and HindIII and ligated with T4 DNA ligase into pLM1-50 (23) previously treated with BsmHI and HindIII. The ligation product was used to transform competent cells of E. coli XL1-blue and the transformants were screened by colony hybridization using the Bio-Rad protein assay kit and bovine serum albumin as standard.

Purification of recombinant trichodiene synthase. Protein concentrations were determined by the method of Bradford (24) using the Bio-Rad protein assay kit and bovine serum albumin as standard.

Fermentation of E. coli BL21(DE3)/pZW03 was halted 3 h after IPTG induction and the cells were harvested by centrifugation at 8000g. The pellet from 500 ml of culture was placed in an 360-ml chamber of a Bead-Beater (Biospec Products, Bartlesville, OK) containing 180 ml of buffer T supplemented with 0.1 mM phenyl methylsulfonyl fluoride and 180 ml of 0.1 mM glass beads. The cells were disrupted using three rounds of 20-s on, 30-s off cycle. The contents of the chamber were then collected and the remaining debris was rinsed with 40–50 ml of buffer T. Any beads which were carried over were removed and the homogenate was centrifuged at 8500g for 10 min. The supernatants were carefully decanted to avoid collecting the lipid, then centrifuged at 150,000g for 65 min. The resulting supernatant was adjusted to 75% saturation ammonium sulfate and the precipitated protein was collected by centrifugation at 20,000g for 15 min. The pellet, which was resuspended in 2 ml of buffer T, could be used directly for the next purification step or stored at −80°C.

The redisolved pellet was desalted by passage through a column of Bio-Gel P-6DG (1.6 × 25 cm) and the protein-containing fractions were directly loaded onto a Q Sepharose anion-exchange column (1.6 × 19 cm). The column was washed with 25 ml of buffer T and then eluted with 200 ml of a linear gradient of 100 to 350 mM KCl in buffer T. Protein eluted in a broad peak between 260 and 310 mM KCl and the highest activity fractions having a trichodiene synthase specific activity of 164 units/mg were pooled. The latter fractions were applied to a column of Superose 12 (1.6 × 45 cm) which was eluted with buffer T. The highest purity fractions were judged to be homogeneous by SDS-PAGE.

N-terminal sequencing. A portion of the Superose 12-purified protein was subjected to SDS-PAGE then transferred to an Immobilon-P PVDF membrane (25) and stained with Coomassie blue. The portion of the membrane carrying the 45,000-Da protein band was cut out and subjected to automated Edman degradation in an ABI Model 477A protein sequencer at the Harvard Microchemistry Facility by Dr. William S. Lane.
RESULTS

To improve the yields of recombinant trichodiene synthase, we initially examined the effect of the cloning host and culture conditions on total cyclase activity. A modest improvement to 0.2% of soluble protein was observed when pTS56-1 was cloned into E. coli XL1-blue and a further twofold increase in yield resulted from lowering the incubation temperature to 25°C following induction with IPTG. On the other hand, the enzyme thus obtained still proved difficult to purify and we therefore explored the use of alternate expression systems. A preliminary examination of several commonly used expression vectors, however, initially gave little or no improvement in synthase expression. Thus subcloning into either pKK223-3 (Pharmacia) (26) or pRT2T (Pharmacia) (27) resulted in levels of trichodiene synthase less than or no greater than those observed for the parent pDR540 clone. No expression at all was observed for the T7-based, T7-7 expression system of Tabor and Richardson (28), while attempted use of the secretion vector pLNIII-1pp-A2 (29) resulted in cell lysis. We therefore turned to the use of the expression cassette PCR (ECPCR) method in which PCR is used to position a strong rbs immediately upstream of the coding sequence of the gene of interest and the resulting amplified construct, containing suitable restriction sites at each terminus, is inserted into an engineered expression vector containing a set of appropriate transcriptional control elements (22). One such recently developed vector is pLM1 (Scheme II), containing a strong T7 promoter and translational leader sequence from T7 gene 10 placed upstream of the pUC19 multiple cloning site which in turn is followed by the E. coli rmBT, T2 tandem transcription terminator (23).

The start primer (Primer 1) for PCR was synthesized so as to contain a BamHI site, a T7 gene 10 rbs and spacer (30), and the first 27 bases of the trichodiene synthase gene, including the normal ATG start codon. The corresponding halt primer (Primer 2) contained a HindIII site, a HindIII sequence followed by the complementary set of bases for the stop codon and eight C-terminal amino acids of trichodiene synthase. Linearized pTS56-1 DNA was used as the template to generate the desired expression cassette by PCR (Scheme 2). The PCR product was digested with BamHI and HindIII, ligated into a similarly digested sample of pLM1, and used to transform competent cells of E. coli XL1-blue. The resulting expression plasmid was designated as pZW03 and was used to transform a suitable expression host, E. coli BL21(DE3), which harbored a prophage carrying the gene for T7 RNA polymerase behind the lacUV5 promoter (30, 31). E. coli BL21(DE3), which lacks the ion protease, was found to be a superior host to the alternative T7 lysogen JM109(DE3). Two colonies from among the resulting transformants were found to have the highest levels of trichodiene synthase activity and one was selected for further study. Sequence analysis of the trichodiene synthase gene in pZW03 confirmed that no base changes had occurred in the PCR amplification and subcloning from the parent pTS56-1 vector.

The time course of trichodiene synthase production was studied under a variety of conditions. Cultures of E. coli BL21(DE3)/pZW03 incubated at 30°C reached a maximum cyclase activity within 3 h after IPTG induction (Fig. 1). The maximum specific activity of the crude extracts corresponded to ca. 20% of the activity of purified trichodiene synthase (see below). The level of trichodiene synthase activity was roughly correlated with the intensity of a 46.3-kDa protein by SDS–PAGE (Fig. 2) which was absent from extracts of cells harboring only the parent expression vector pLM1. Western blot analysis using specific antiserum to the F. sporotrichioides synthase confirmed the identity of this band (data not shown) as trichodiene synthase. Laser densitometric analysis of stained SDS–PAGE gels run on cells boiled in SDS loading buffer led to an estimate that this band corresponded to 30% of total cellular protein.

The recombinant synthase from a 500-ml culture was readily purified fivefold to homogeneity by a two-step procedure involving ion-exchange chromatography on Q Sepharose of the 75% ammonium sulfate pellet followed by gel filtration on Superose 12 to remove a very minor 66-kDa protein contaminant (Table I). The overall yield of the purification procedure was 12%, corresponding to 6 mg of purified protein from 500 ml of culture. The homogenous synthase had a measured $V_{max}$ of 169 units/mg, compared to a previously determined $V_{max}$ for the native fungal enzyme of 102 units/mg (12). The measured $K_m$ for recombinant cyclase was 83 ± 4 nM, in close agreement with the value of 75 ± 4 nM for the native enzyme (8). N-terminal sequencing of the recombinant protein gave a sequence of MENFP, in complete agreement with the established DNA sequence and consistent with the previously observed N-terminal ENFP sequence of the native fungal enzyme.

DISCUSSION

Although by now the structural genes for several sesquiterpene cyclases have been cloned and sequenced (32), prior to the current work only one, trichodiene synthase, had been reported to be expressed in a bacterial host (15). Unfortunately, the maximum levels of cyclase expression achieved with the originally reported construct, pTS56-1 (0.05–0.1% of total protein) (15), were actually lower by a factor of more than 10 than those previously observed for high-producing cultures of F. sporotrichioides (12). We now have achieved the expression of trichodiene synthase at levels ranging from 20 to 30% of soluble protein. Examination of cell lysates for insoluble components by SDS–PAGE indicated insignificant amounts of protein with a mobility corresponding to the characteristic 46.3-kDa trichodiene synthase band. This observation is in
contrast to the formation of large amounts of inclusion bodies which frequently accompanies or even dominates attempts to attain high-level overexpression in bacterial hosts.

The success of the expression strategy described above probably rests on the interaction of several important factors. By placing the trichodiene synthase gene under the control of the T7 promoter, one can simultaneously suppress unwanted expression by the RNA polymerase of the host organism, XL1-blue, in primary transformants, while allowing expression from a strong promoter in hosts harboring an inducible T7 RNA polymerase system (28, 30, 31). In this regard, the reasons for the failure of attempts to express trichodiene synthase with the widely used T7-7 expression system are unclear. Although the precise influence of the T7 gene 10 translational leader sequence in pLM1 is uncertain, it is likely that the mRNA for this highly expressed gene product is exceptionally stable. Similarly, the T7 gene 10 rbs and spacer introduced in the expression cassette has previously been shown to support high-level expression in pLM1 and related systems (22, 23, 33). Interestingly, insertion of the same PCR-generated trichodiene synthase expression cassette into two related vectors with different promoters and transcriptional sequences, pHN1⁺ (tac promoter) (22) and pKEN2 (src promoter),⁴ resulted in extremely low expression of the desired synthase activity. Finally, the greater observed levels of production of trichodiene synthase in E. coli BL21(DE3)/pZW03 in comparison to those of JM109(DE3)/pZW03 may reflect the absence of the lon protease in the former host, resulting in greater stability of the protein product.

The recombinant trichodiene synthase could be purified by a convenient two-step procedure. The N-terminal se-

⁴ G. Verdine, personal communication.
OVERPRODUCTION OF TRICHODIENE SYNTHASE IN Escherichia coli

TABLE I

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>150,000g supernatant</td>
<td>277</td>
<td>8600</td>
<td>31</td>
<td>-</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ precipitation</td>
<td>100</td>
<td>5600</td>
<td>55</td>
<td>65</td>
</tr>
<tr>
<td>Q Sepharose</td>
<td>11</td>
<td>1800</td>
<td>164</td>
<td>21</td>
</tr>
<tr>
<td>Superose 12</td>
<td>6.1</td>
<td>1031</td>
<td>169</td>
<td>12</td>
</tr>
</tbody>
</table>

*Data are based on protein from a 500-ml culture.

fungal enzyme in gel mobility and steady-state kinetic parameters. The small increase in observed $V_{\text{max}}$ for the recombinant synthase probably reflects differences in the particular protein standards used to determine protein concentration. Trichodiene synthase is now available in substantial quantities and is currently being used in studies designed to identify the nature of the active site and overall protein structure of this important cyclase.

ACKNOWLEDGMENTS

We thank Professor Gregory L. Verdine for a gift of the expression vector, pLM1, and for helpful discussions, and Mr. Guohan Yang of Brown University for carrying out the determination of steady-state kinetic parameters.

REFERENCES


FIG. 1. Time dependence of trichodiene synthase activity of *E. coli* BL21(DE3)/pZW03 as a function of time after IPTG induction and incubation at 30°C. Assays were carried out on 10-μl aliquots of cell lysate for 15 min at 30°C in the presence of 1.13 × 10⁵ dpm of [1-³H]FPP as described under Experimental Procedures. Activity refers to activity of hexane elutable product.

FIG. 2. Ten percent SDS-PAGE analysis of trichodiene synthase production: (molecular weight standards in kDa). Lane 1, total cell protein from BL21(DE3)/pLM1; Lanes 2-4, total cell protein of BL21(DE3)/pZW03 1.5, 2.5, and 3.5 h after IPTG induction (30°C); Lane 5, supernatant after 150,000g centrifugation of extracts (Table I); Lane 6, pellet after 75% ammonium sulfate precipitation; Lane 7, pooled fractions from Q Sepharose column; Lane 8, pooled fractions from Superose 12 column.

sequence of the homogenous protein was identical to that of the native synthase save for the additional Met at the N-terminus. Purified recombinant trichodiene synthase was also essentially indistinguishable from the native product.

Supplied by U.S. Dept. of Agric., National Center for Agricultural Utilization Research, Peoria, IL