Isolation and bacterial expression of a sesquiterpene synthase
cDNA clone from peppermint (Mentha x piperita, L.) that
produces the aphid alarm pheromone (E)-β-farnesene

John Crock, Mark Wildung, and Rodney Croteau*

Institute of Biological Chemistry, and Department of Biochemistry and Biophysics, Washington State University, Pullman, WA 99164-6340

Contributed by Rodney Croteau, September 25, 1997

ABSTRACT (E)-β-Farnesene is a sesquiterpene semiochemical that is used extensively by both plants and insects for communication. This acyclic olefin is found in the essential oil of peppermint (Mentha x piperita) and can be synthesized from farnesyl diphosphate by a cell-free extract of peppermint secretory gland cells. A cDNA from peppermint encoding (E)-β-farnesene synthase was cloned by random sequencing of an oil gland library and was expressed in Escherichia coli. The corresponding synthase has a deduced size of 63.8 kDa and requires a divalent cation for catalysis ($K_m$ for Mg$^{2+}$ ~ 150 μM; $K_m$ for Mn$^{2+}$ ~ 7 μM). The sesquiterpenoids produced by the recombinant enzyme, as determined by radio-GC and GC-MS, are (E)-β-farnesene (85%), (Z)-β-farnesene (8%), and δ-cadinene (5%) with the native C15 substrate farnesyl diphosphate ($K_m$ = 0.6 μM; $V_{rel}$ = 100) and Mg$^{2+}$ as cofactor, and (E)-β-farnesene (98%) and (Z)-β-farnesene (2%) with Mn$^{2+}$ as cofactor ($V_{rel}$ = 80). With the C19 analog, GDP, as substrate ($K_m$ = 1.5 μM; $V_{rel}$ = 3 with Mg$^{2+}$ as cofactor), the monoterpenes limonene (48%), terpinolene (15%), and myrcene (15%) are produced.

(E)-β-farnesene (Fig. 1) is an acyclic sesquiterpene olefin that occurs in a wide range of both plant and animal taxa. More than 600 papers have been published on the occurrence of this sesquiterpene and its deployment as an important courier in both plant-insect and plant-plant chemical communication. The olefin is found in the essential oil of hundreds of species of both gymnosperms, such as Torrey taxifolia (Florida torrey) (1) and Larix leptolepis (larch) (2), and angiosperms, such as Robinia pseudoacacia (black locust) (3), Medicago sativa (alfalfa) (4), Chamomilla recutita (chamomile) (5), Vitis vinifera (grapes) (6), Cannabis sativa (hemp) (7), Zea mays (corn) (8), Piper nigrum (black pepper) (9), Daucus carota (carrot) (10), and Mentha x piperita (peppermint) (9).

Although socially dominant male mice produce both α-farnesene and (E)-β-farnesene in their urine as pheromones (10), it is in the insects and plants that the use of (E)-β-farnesene as a semiochemical is most extensive. (E)-β-Farnesene is emitted by the Dufour’s gland of andrenid bees (11) and by several genera of ants (12-14), where it serves both as a defensive allomone and as a trail pheromone. This sesquiterpene is synthesized de novo in the esomtral glands of larval Papilio (Lepidoptera/Papilionidae) as an allomone (15), and it functions as a feeding stimulant to the sand fly Lutzomyia longipalpis (Diptera/ Psychodidae), an important vector of the blood disease leishmaniasis (16). Several species of predatory carabid beetles use (E)-β-farnesene as a prey-finding kairomone (17). When released by corn, this olefin is also a kairomonal oviposition stimulant to the European corn borer (Ostrinia) (18). (E)-β-farnesene is the major component of pollen odor in Lupinus and stimulates pollination behavior in bumblebees (19). Feeding by larval lepidopterans, such as Heliotris or Spodoptera (Noctuidae), increases the amount of (E)-β-farnesene released by corn; the volatile olefin then is detected as a synomone by the parasitic wasp Cotesia marginiventris (Hymenoptera/Bracidae) for locating the lepidopteran hosts (8). Circumstantial evidence also suggests the lepidopteran induced production and emission of (E)-β-farnesene from corn serves as a synomone for Cotesia kariyai (20) and from cotton leaves as a synomone for C. marginiventris (21, 22).

Perhaps of greatest significance in plant-insect interactions is the use of (E)-β-farnesene by most aphid species as an alarm pheromone (23, 24). Aphids exposed to (E)-β-farnesene become agitated and disperse from their host plant (25). Alate aphids are usually more sensitive than are apterae species and often will not colonize a host displaying (E)-β-farnesene. Ants that defend aphids are sensitive to host-emitted (E)-β-farnesene and, when exposed, will display aggressive behavior (26). (E)-β-farnesene also mimics the action of juvenile hormone III in some insects (27), may play a role in control of aphid morphological types, and is acutely toxic to aphids at a dose of 100 ng/aphid (28). (E)-β-farnesene vapor is also toxic to whiteflies (29).

Efforts to control aphid behavior by topical application of (E)-β-farnesene to crops have met with little success, because of volatility and rapid oxidative inactivation in air (30). Derivatives of (E)-β-farnesene with reduced volatility, or increased stability, have shown promise in reducing aphid-transmitted viruses, such as barley mosaic virus (30), potato virus Y (31), and beet mosaic virus (31). The wild potato Solanum berthaultii, which produces (E)-β-farnesene in type A trichomes, is more repellent to the green peach aphid than are commercial varieties of S. tuberosum that produce lower levels of the olefin (32, 33). In alfalfa, repellency to the blue alfalfa aphid and the pea aphid is correlated with the leaf content of (E)-β-farnesene, but not with the amount of the co-occurring sesquiterpene caryophyllene (34).

For plants that produce (E)-β-farnesene, breeding for increased production has met with some success (34), but has been limited by genetic variation in these species. (E)-β-farnesene synthase has been purified from maritime pine (Pinus pinaster) and characterized (35), but the gene has not yet been isolated from any source. A cDNA clone for (E)-β-

Abbreviations: FDP, farnesyldiphosphate; I, identity; S, similarity.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF804615).

To whom reprint requests should be addressed. e-mail: croteau@ mail.wsu.edu.

© 1997 by The National Academy of Sciences 0027-8424/97/9412833-6$2.00
PNAS is available online at http://www.pnas.org.
MATERIALS AND METHODS

Plant Material and Reagents. Mentha x piperita L. cv. “Black Mitcham” was propagated from rhizomes as described (37). The preparations of \([1-^{3}H] GDP \) (250 Ci/mol), \([1-^{3}H] FDP \) (125 Ci/mol), and \([1-^{3}H] \text{geranylgeranyl } diphosphate \) (118 Ci/mol) have been reported previously (38–40). Terpenoid standards were from our own collection or were prepared from plant material purchased locally. \(\alpha\)-Farnesene was a gift from J. Brown (Washington State University), \(\delta\)-cadinene was a gift from M. Eisenberg (Oklahoma State University), and commercially steam distilled peppermint oil was a gift from I. P. Callison and Sons, Inc., Chehalis, WA. All other biochemicals and reagents were purchased from Sigma or Aldrich unless otherwise noted.

Sesquiterpene Analysis. Young, mature peppermint leaves were harvested and hydrodistilled from \(\text{NH}_4\text{HCO}_3\)-buffered water with simultaneous pentane extraction (41). The organic phase was passed through a column of anhydrous \(\text{MgSO}_4\)-silica gel column to obtain the labeled hydrocarbon fraction for GC-MS analysis, or for radio-GC analysis by using peppermint oil as an internal standard.

Instrumental Analysis. Radio-GC was performed on a Gow-Mac 550P instrument (He carrier 40 ml/min, injector 220°C, detector 250°C and 150 mA) attached to a Packard 894 gas proportional counter. The column (3.18 mm i.d. by 3.66 m stainless steel with 15% polyethylene glycol ester (AT1000 Alltech) on Gas Chrom Q was programmed from 150°C (5-min hold) to 220°C at 5°C/min. Thermal conductivity and radioactivity outputs were monitored after calibration with an external radiochemical standard, and \(\sim 20,000\) disintegrations per minute of tritiated product were injected with data analysis that used Turbochrome Navigator version 4.1 software (Perkin-Elmer). Liquid scintillation counting was performed in toluene/ethanol (70:30, vol/vol) containing 0.4% Omnifluor (DuPont/ NEN) by using a Packard 460 CD spectrometer (\(^{3}H\) efficiency \(\sim 43\%)\). GC-MS analysis used a Hewlett-Packard 6890–5972 system with a 5MS capillary column (0.25 mm i.d. by 30 m with 0.25 \(\mu\)m coating of 5% phenyl methyl siloxane). Injections were made cool on-column at 40°C with oven programming from 40°C (50°C/min) to 70°C (5°-min hold), then 10°C/min to 250°C, then 50°C/min to 300°C. Separations were made under a constant flow of 0.7 ml He/min. Mass spectral data were collected at 70 eV and analyzed by using Hewlett–Packard Chemstation software.

Library Construction and Clone Identification. An enriched cDNA library was constructed from peppermint secretory cell clusters consisting of the eight glandular cells subduing the oil droplet. These cell clusters were harvested by a leaf surface abrasion technique (37), and the RNA contained therein was isolated by using the protocol of Logemann et al. (42). mRNA was purified by oligo(dT) cellulose chromatography (Pharmacia), and 5 \(\mu\)g of mRNA was used to construct a \(\alpha\)-ZAPII cDNA library according to the manufacturer’s instructions (Stratagene).

Plasmids were excised from the library en masse and used to transform \(E\). \(\text{coli}\) strain XLOLR as per the manufacturer’s instructions (Stratagene). Approximately 150 individual plasmid-bearing strains were grown in 5 ml of Luria–Bertani media overnight, and the corresponding plasmids were purified by using a Qiawell 8 Ultraplasmid Kit (Qiagen) before partial 5′-sequencing by the Dye-Deoxy method by using an Applied Biosystems Sequenator at the Laboratory for Biotechnology and Bioanalysis at Washington State University. Putative terpenoid synthase genes were identified by sequence comparison by using the BLAST program of the GCG Wisconsin Package, version 8. Bluescript plasmids harboring unique full-length cDNA inserts with high similarity to known plant terpenoid synthases were tested for functional expression after transformation into \(E\). \(\text{coli}\) XL1-Blue cells. A single extract, from the bacteria containing clone p43, produced a sesquiterpene olefin from \([1-^{3}H] FDP\), and this clone was selected for further study.

Bacterial Expression and Characterization of \((E)-\beta\)-Farnesene Synthase. \(E\). \(\text{coli}\) XL1-Blue harboring p43 (bearing an apparently full-length insert) or empty pBluescript plasmid as a control were grown overnight at 37°C in Luria–Bertani
RESULTS AND DISCUSSION

Essential Oil Analysis and Cell-Free Assay. To assess the probable abundance of (E)-β-farnesene synthase in peppermint gland secretory cells, the exclusive site of essential oil biosynthesis (37), the sesquiterpene olefin fraction of field-distilled peppermint oil was analyzed by GC-MS and shown to contain β-caryophyllene (39%), γ-cadinene (33%), β-bourbonene (11%), (E)-β-farnesene (2.9%), δ-cadinene (2.0%), germacrene D (1.3%), copaene (1.3%) and α-humulene (1.2%) (Fig. 1), as well as several other minor components (<1% each). GC-MS analysis of the oil distilled from greenhouse material revealed a similar composition, except that the amount of γ-cadinene was higher (53%), β-bourbonene was conspicuously absent, and the (E)-β-farnesene content was 3.4%. Although (E)-β-farnesene was not one of the more prominent sesquiterpenes of peppermint, the abundance was sufficient to suggest that cloning of the corresponding synthase by random sequencing of an enriched, oil gland cDNA library might be possible.

To gain a preliminary assessment of the target activity, cell-free extracts of peppermint oil gland secretory cells (37) were assayed for the divalent metal-ion-dependent conversion of [1-3H]FDP to labeled sesquiterpene olefins (44). Radio-GC analysis of the derived biosynthetic products (Fig. 2) revealed the presence of two major components identified as caryophyllene and γ-cadinene. However, the separation of the labeled olefins was insufficient to resolve (E)-β-farnesene from caryophyllene, or δ-cadinene from γ-cadinene. Both of these minor components appear at the trailing edges of the major peaks but are, nevertheless, coincident with the authentic standards, indicating the corresponding biosynthetic capability. No β-bourbonene was synthesized from FDP by this system.

Isolation and Bacterial Expression of a cDNA Encoding (E)-β-Farnesene Synthase. Initial cloning of full-length terpenoid biosynthetic genes from the peppermint oil gland cDNA library was successful and established a very high degree of enrichment for these target sequences. For example, the monoterpene cyclase, limonene synthase (45), represents approximately 4% of the library. This fact, plus the availability of automated sequencing capability, led to the possibility of randomly sequencing the library in search of cDNA species encoding other terpenoid synthases, including the (E)-β-farnesene synthase, which was shown to be operational in this plant by both sesquiterpene analysis and cell-free assay.

Of the first 150 random clones, a unique and apparently full-length insert was acquired that was shown by partial sequencing to resemble, in deduced amino acid sequence, other sesquiterpene synthases of plant origin (i.e., a similarity score in excess of 50%, see below). A cell-free extract of E. coli XL-1 Blue cells harboring this plasmid (p43) was prepared and shown to be capable of catalyzing the divalent metal ion-dependent conversion of [1-3H]FDP to labeled sesquiterpene olefins. Radio-GC analysis (data not shown) and GC-MS analysis (Fig. 3) of this sesquiterpene olefin fraction demonstrated that the major biosynthetic product (85%) was (E)-β-farnesene by matching of both retention time and mass spectrum to those of the authentic standard obtained from several natural sources. Lesser amounts of (Z)-β-farnesene (8%) and δ-cadinene (5%), as well as three other minor products (less than 1% each; all seemingly of the cadinene-type based on MS) were also produced. Control reactions, by using extracts of XL1-Blue cells transformed with pBluescript lacking the insert, evidenced no detectable production of sesquiterpene olefins from [1-3H]FDP, thereby demonstrating that a cDNA clone encoding (E)-β-farnesene synthase had been acquired.

Multiple product formation is a common feature of the terpenoid synthases and may be a consequence of the electrophilic reaction mechanism catalyzed by these enzymes in which highly reactive carbocationic intermediates are generated (44, 46). (E)-β-Farnesene is one of the simplest sesquiterpene olefins that can be derived from FDP, in a reaction

FIG. 2. Radio-GC of the sesquiterpene olefins generated from [1-3H]FDP by an enzyme preparation from peppermint oil gland secretory cells. The olefin fraction of steam-distilled peppermint oil was used as internal standard, and only the portion of the chromatogram containing the sesquiterpene olefins is shown.
involving divalent metal ion-assisted ionization of the diphosphate ester and deprotonation from the C-3 methyl of the resulting carbocation (Fig. 4). The formation of δ-cadinene (Fig. 4) involves a considerably more extended reaction sequence, in which a preliminary isomerization step (to nerolidyl diphosphate) is required to permit the ionization-dependent cyclization to the macrocycle, followed by 1,3-hydride shift, closure of the second ring, and deprotonation to the bicyclic product. The small amount of δ-cadinene produced by the recombinant synthase from FDP is interesting in light of the abundance of this bicyclic olefin in the sesquiterpene fraction of peppermint oil and the efficient production of this olefin in oil gland extracts; these observations suggest that an additional and distinct δ-cadinene synthase must operate in peppermint.

The recombinant (E)-β-farnesene synthase was inactive with the C_{20} substrate analog [1-3H]geranylgeranyl diphosphate, but was able to catalyze the divalent cation-dependent conversion of the C_{10} analog [1-3H]GDP to monoterpene olefins. Although the rate of conversion of GDP to these products was less than 3% of the rate of conversion of FDP to sesquiterpene olefins at saturation, a more diverse spectrum of products was formed (see Fig. 5 for structures). The cyclic monoterpene limonene (48%) and terpinolene (15%) and the acyclic monoterpene analog of β-farnesene, myrcene (15%), were the most abundant products as determined by both radio-GC and GC-MS analysis (data not shown). Lesser amounts of γ-terpinene (7%), (Z)-ocimene (6%), (E)-ocimene (7%), and sabinene (3%) also were observed as products. Control reactions, by using extracts of XL1-Blue cells transformed with pBluescript lacking the insert, showed no detectable production of monoterpene olefins from [1-3H]GDP, thereby confirming that the monoterpene synthase activity expressed from p43 was a function of the (E)-β-farnesene synthase. Because monoterpene biosynthesis is localized to plastids, as is diterpene biosynthesis, whereas sesquiterpene biosynthesis occurs in the cytoplasm (36), the utilization of GDP as a substrate by (E)-β-farnesene synthase is unlikely to be of physiological relevance and simply may reflect the lack of evolutionary pressure to discern the chain length of this isoprenoid substrate to which the enzyme is not exposed in vivo.

Sequence Analysis. Complete sequencing of the (E)-β-farnesene synthase cDNA contained in p43 revealed an insert

![Detector Response (1000's of ions)]

![Relative Abundance]

![Fig. 3. GC-MS of the products generated from FDP by the recombinant (E)-β-farnesene synthase. (A) Total ion chromatogram. Numbered peaks are sesquiterpene olefins. (B) Mass spectrum and retention time of peak 1 designated in A. (C) Mass spectrum and retention time of authentic (E)-β-farnesene from parley oil. (D) Mass spectrum and retention time of peak 6 designated in A. The spectrum of this minor product is compromised by the low ion abundance and the corresponding prominence of background ions. (E) Mass spectrum and retention time of authentic δ-cadinene.]

![Fig. 4. Proposed mechanism for the formation of (E)-β-farnesene and δ-cadinene from FDP. OPP denotes the diphosphate moiety. Ionization of the enzyme-bound nerolidyl diphosphate intermediate and proton elimination also can produce (E)-β-farnesene.]

![Fig. 5. Monoterpene olefins generated from the alternate substrate GDP by recombinant (E)-β-farnesene synthase.]

Geranyl diphosphate
Limene
Terpinolene
γ-Terpinene
Sabinene
Myrcene
Z-Ocimene
E-Ocimene
size of 1,959 bp encoding an ORF of 550 amino acids with a deduced molecular mass of 63,829 (Fig. 6). A putative starting methionine codon was identified that was out of frame with the vector β-galactosidase starting methionine; however, a fortuitous stop codon in the 5'-untranslated region, 46 bp upstream of the synthase translation start site and in-frame with the β-galactosidase fusion sequence, allowed polycistronic translation of the cDNA free of vector-derived sequence. The deduced amino acid sequence of the (E)-β-farnesene synthase lacks a plastidial targeting peptide (47), typical of monoterpenes and diterpene syntheses (45, 48, 49), but consistent with all known plant-derived sesquiterpene syntheses (50–52) that are directed to the cytoplasm. Like all other known terpenoid syntheses, (E)-β-farnesene synthase is rich in tryptophan (1.8%) and arginine (5.5%) residues, and bears a DDXXD motif (residues 301–305), which is believed to coordinate the divalent metal ion chelated to the substrate diphosphate group (53); the enzyme has a deduced isoelectric point at pH 5.16.

The deduced amino acid sequence of the farnesene synthase is most similar to that of the sesquiterpene cyclase epimorpholino)-2-hydroxypropan-1-ol added to the 1-amylopectin moiety of the monosaccharide chain, resulting in a branched polysaccharide with a total chain length of about 200 monosaccharide units. The polysaccharide has a high molecular weight, typically in the range of 10,000 to 100,000.

**Enzyme Characterization.** The recombinant, partially purified (E)-β-farnesene synthase exhibited a broad pH optimum in the 6.75 to 7.25 range in 3-(N-morpholino)-2-hydroxypropane-sulfonic acid buffer. This observation is in agreement with the studies of Salin et al. (35) in which the purified (E)-β-farnesene synthase from maritime pine was shown to possess a pH optimum in the 7.0 to 7.3 range. The Km value for FDP with the recombinant synthase was calculated to be 0.6 μM, a value typical of other sesquiterpene syntheses of plant origin (44) but lower than the value of 5 μM determined for the enzyme from maritime pine (35). Substrate concentrations in excess of 10 μM FDP indicated slight inhibition of activity with the recombinant enzyme. Although the relative velocity at saturating levels of GDP was only 3% of the velocity with FDP for the recombinant synthase, the calculated Km value for GDP (1.5 μM) was only 3-fold higher than that for FDP, suggesting that the binding of the C1α analog was reasonably efficient.

A Km value of 150 μM was determined for Mg2+ (Vrel = 100), and a Km value of 7.0 μM was determined for Mn2+ (Vrel = 80). No inhibition of activity was observed at Mg2+ concentrations up to 10 mM; however, concentrations of Mn2+ exceeding 20 μM resulted in a sharp decline in reaction velocity to a plateau (Vrel = 20) in the 0.25 to 2 mM range. Because the product distribution of the recombinant (E)-β-farnesene synthase from maritime pine was determined in the presence of excess Mg2+, the conversion of [1-3H]FDP was re-evaluated in the presence of Mn2+ alone at apparent saturation (20 μM). The olefin products again were analyzed by GC-MS and found in this case to consist of 98% (E)-β-farnesene and 2% (Z)-β-farnesene. No δ-cadinene or other sesquiterpenes were synthesized in this instance, indicating that a structural alteration in the binding of Mn2+ to the substrate and/or enzyme (relative to Mg2+) improves the fidelity of the reaction.

In operational characteristics (pH optimum, kinetic constants) and physical features (size, pi), the recombinant (E)-β-farnesene is a typical sesquiterpene synthase (44, 50–52), suggesting that the enzyme should be highly functional in planta. Given that this synthase will be targeted by default to the cytoplasm (36, 47), where the substrate arises from the mevalonate pathway, it should be possible to engineer virtually any plant for the production of (E)-β-farnesene to exploit the kainomonal and phenomeral properties of this natural product.

This work was supported by National Institutes of Health Grant GM-31354 and Project 0268 from the Agricultural Research Center, Washington State University.