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Cloning, expression, purification and characterization of recombinant (+)-germacrene D synthase from *Zingiber officinale*

Sarah Picaud, Mikael E. Olsson, Maria Brodelius, Peter E. Brodelius *

Department of Chemistry and Biomedical Sciences, University of Kalmar, SE-39182 Kalmar, Sweden

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Abstract

A cDNA clone encoding a sesquiterpene synthase, (+)-germacrene D synthase, has been isolated from ginger (*Zingiber officinale*). The full-length cDNA (AY860846) contains a 1650-bp open reading frame coding for 550 amino acids (63.8 kDa) with a theoretical p$I = 5.59$. The deduced amino acid sequence is 30–46% identical with sequences of other sesquiterpene synthases from angiosperms. The recombinant enzyme, produced in *Escherichia coli*, catalyzed the formation of a major product, (+)-germacrene D (50.2% of total sesquiterpenoids produced) and a co-product, germacrene B (17.1%) and a number of minor by-products. The optimal pH for the recombinant enzyme is around 7.5. Substantial (+)-germacrene D synthase activity is observed in the presence of Mg^{2+}, Mn^{2+}, Ni^{2+} or Co^{2+}, while the enzyme is inactive when Cu^{2+} or Zn^{2+} is used. The K_m- and k_{cat}-values are 0.88 μM and $3.34 \times 10^{-3}\ s^{-1}$, respectively. A reaction mechanism involving a double 1,2-hydride shift has been established using deuterium labeled substrates in combination with GC–MS analysis.

Keywords: cDNA cloning; Enzyme characterization; (+)-Germacrene D synthase; Recombinant protein; Sesquiterpenes; *Zingiber officinale*

The Chinese have long valued ginger (*Zingiber officinale* Roscoe) to promote strength and to ensure a long life. It was one of the first products to travel the "spice route" from Asia to Europe, where Greeks and Romans used it extensively in their pharmacopoeias as well as spice, aromatic and food. The characteristic pleasant and aromatic odour of ginger is due to various mono- and sesquiterpenoids, which can be isolated from the essential oil. The vast majority of terpenoids are classified as secondary metabolites, compounds not required for plant growth and development but presumed to have an ecological function in communication and defence [1]. They display a very wide range of diversity in carbon skeletons, from relatively simple linear carbon chain to highly complex ring structures, and functional groups. Individual plants can contain as much as 100 different terpenes as components of complex oils, resins or volatile mixture [2]. Ginger contains gingerol, a ginger oleoresin (combination of volatile oils and resin) that accounts for the characteristic aroma of ginger, and explains its therapeutic properties. Components of gingerol (zingiberone, bisabolene, camphene, geranial, linalool and borneol) have recently been studied and found to possess beneficial properties for the treatment of poor digestion, heartburn, vomiting and preventing motion sickness. One sesquiterpene, zerumbone from tropical ginger (*Zingiber zerumbet*), has been implicated in one of the promising chemo-preventive agents against colon and skin cancer [3]. Zingiberene, one of the major compounds found in ginger rhizome, has an anti-viral, anti-ulcer and anti-fertility effect. It has been also widely used in cosmetics and fragrances [4]. Despite its importance for human health care, nothing is known about the enzymes producing these compounds *in planta*.

Sesquiterpene synthases are a ubiquitously expressed family of proteins capable of converting the universal acyclic precursor farnesyl diphosphate (FPP)[1] into more than 300

* Corresponding author. Fax: +46 480 446262.
E-mail address: peter.brodelius@hik.se (P.E. Brodelius).

[1] *Abbreviations used:* FPP, farnesyl diphosphate; GPP, geranyl diphosphate; GS, (+)-germacrene D synthase; IMAC, immobilized metal affinity chromatography; IPTG, isopropyl-β-D-thiogalacto-pyranoside; RACE, rapid amplification of cDNA ends.

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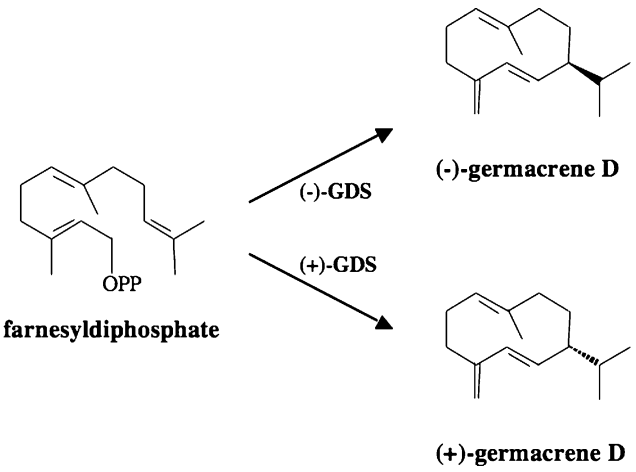

Fig. 1. Reaction catalyzed by (−)- and (+)-germacrene D synthase.

different sesquiterpene skeletons [5]. This diversity requires the evolution of numerous enzymes with different product specificities. This is the first communication on the isolation and characterization of a sesquiterpene synthase from *Zingiber officinale*, a germacrene D synthase. Germacrene D is a chiral compound existing in two enantiomers, which are produced from FPP by enantiomer-specific synthases (Fig. 1) [6]. Germacrene D has a strong effect on insect behaviour. Germacrene D synthases (GDS) have previously been cloned from a number of dicotylodonous plants including *Solidago canadiensis* [6], *Lycopersicon hirsutum* [7], *Vitis vinifiera* [8], *Rosa hybrida* [9] and *Ocimum basilicum* [10].

Materials and methods

Reagents

Restrictions enzymes *Nhe*I, *Not*I and the T4 DNA ligase were purchased from New England Biolabs. *Pyrobest*® DNA polymerase and the corresponding buffer were supplied by Takara Bio Inc. Sequencing was performed by MWG Germany (http://www.mwgdna.com). HiTrap Chelating HP PD-10 columns, Sephadex™ G-25M and [1-^{3}H]FPP (0.59 TBq/mmol) were from Amersham Biosciences and [1-^{3}H]GPP (0.56 TBq/mmol) from American Radiochemical Co. [1*R*-^{2}H]FPP, [1*S*-^{2}H]FPP and [1,1-$^2H_2$]FPP were kindly supplied by D. Cane and Ms. Xin Lin, Brown University. IPTG was obtained from Saveen Werner AB. FPP (dissolved in methanol: 10 mM aqueous NH_4OH (7:3)), GPP (dissolved in methanol: 10 mM aqueous NH_4OH (7:3)) and kanamycin were obtained from Sigma. All other biochemicals and reagents were purchased from commercial sources.

RNA extraction

Ginger rhizomes (*Zingiber officinale* Roscoe) from Thailand were purchased from a local supermarket. Buds were allowed to develop until the sheath reach 15 cm high. Then the small rhizome formed at the base of the sheath was cut off the plant and frozen in liquid nitrogen. Because of the high content in starch, a phenol RNA extraction protocol was used. Two grams of frozen tissue were ground in liquid nitrogen and mixed with 7.5 mL of extraction buffer [1 M Tris–HCl, pH 7.5; 10 mM EDTA; SDS 1% (w/v)] and 1 vol of phenol/chloroform/isoamylic alcohol [25:24:1 (v/v/v)]. The mixture was homogenized 15 min at 4 °C. After centrifugation [10 min, 10,000*g*, 4 °C] the nucleic acids were precipitated during 20 min at −20 °C with 1/10 vol of 3 M sodium acetate, pH 5.2, and 2 vol of 100% ethanol. Pelleted nucleic acids were re-suspended in 1 mL of ultra pure water. RNA was selectively precipitated over night at 4 °C with 4 M LiCl. Total RNA was re-suspended in ultra-pure water. DNaseI treatment (Amersham Biosciences) was performed to remove the last traces of DNA. Total RNA was quantified spectrophotometrically.

Oligonucleotide primer design

Comparison of the amino acid sequences from sesquiterpene synthases isolated from divergent sources revealed highly conserved boxes. These sequences were back translated and degenerate primers were designed. One forward primer (5′-TAGATGACACWTWTGATGCTTAYGGT AYTTWGAA-3′) and one reverse primer (5′-AGGIAYT CCBRTTTATATCYTTCCATGC-3′) (R = A or G; Y = C or T; W = A or T; B = G or T or C) were selected with respective annealing temperature of 66.1 and 61.2 °C calculated with [$Tm = 69.3 + GC\% \times 0.41 - 650/(nA + nC + nG + nT)$].

Cloning of the germacrene D synthase cDNA by RT-PCR

The first strand cDNA synthesis was initiated on 5.0 μg of total RNA at the poly(A)-tail of mRNA using the adapter primer (AP) and the Superscript II reverse transcriptase (Invitrogen). After the first strand cDNA synthesis, the mRNA was degraded with RNase H (Invitrogen). This first strand reaction was used for PCR (PuReTaq Ready-To-Go PCR beads, Amersham Biosciences) with the two degenerate primers. The hot start conditions were: 94 °C for 2 min and then, 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min for a total of 30 cycles, followed by a final extension step at 72 °C for 5 min. The obtained DNA fragment with a length of 588 bp was electrophoresed through a 1% agarose gel. After gel purification, the PCR fragment was cloned into the plasmid vector pGEM®-T Easy (Promega). The plasmid obtained was transformed into competent *Escherichia coli* NovaBlue cells according to standard procedures. The cells were selected on ampicillin plates (50 μg/mL) complemented with X-Gal and IPTG. The resulting plasmid DNA was sequenced. A 5′ rapid amplification of cDNA ends (5′ RACE PCR) was performed with gene-specific primers designed against the partial sequence obtained through RT-PCR with the two degenerated primers and according to the manufacturer instructions.