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Expression, purification and characterization of recombinant (*E*)-β-farnesene synthase from *Artemisia annua* ☆

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Abstract

A cDNA clone (GenBank Accession No. AY835398) encoding a sesquiterpene synthase, (*E*)-β-farnesene synthase, has been isolated from *Artemisia annua* L. It contains a 1746-bp open reading frame coding for 574 amino acids (66.9 kDa) with a calculated p*I* = 5.03. The deduced amino acid sequence is 30–50% identical with sequences of other sesquiterpene synthases from angiosperms. The recombinant enzyme, produced in *Escherichia coli*, catalyzed the formation of a single product, β-farnesene, from farnesyl diphosphate. The pH optimum for the recombinant enzyme is around 6.5 and the K_m- and k_{cat}-values for farnesyl diphosphate, is 2.1 μM and 9.5×10^{-3} s^{-1}, respectively resulting in the efficiency 4.5×10^{-3} M^{-1} s^{-1}. The enzyme exhibits substantial activity in the presence of Mg^{2+}, Mn^{2+} or Co^{2+} but essentially no activity when Zn^{2+}, Ni^{2+} or Cu^{2+} is used as cofactor. The concentration required for maximum activity are estimated to 5 mM, 0.5 mM and <10 μM for Mg^{2+}, Co^{2+} or Mn^{2+}, respectively. Geranyl diphosphate is not a substrate for the recombinant enzyme.

Keywords: *Artemisia annua*; β-Farnesene synthase; cDNA cloning; GC–MS; Recombinant expression; Sesquiterpenes

1. Introduction

The aerial parts of the plant *Artemisia annua* L. (Asteraceae) (sweet wormwood) has during centuries been used in traditional Chinese medicine (qinghao) to treat various fever diseases. The plant produces the endoperoxide sesquiterpene lactone antimalarial drug artemisinin (qinghaosu). Following the discovery of artemisinin, much phytochemical work has been undertaken with *A. annua*. A number of sesquiterpenoids have been isolated from leaves of *A. annua*. α-Copaene, β-caryophyllene, *trans*-β-farnesene, muurola-4,11-diene, selina-4,11-diene, germacrene D, β-selinene, bicyclogermacrene, germacrene A, γ-cadinene, α-humulene, and amorpha-4,11-diene were identified in solvent extracts of greenhouse-grown *A. annua* leaves by GC–MS (Bouwmeester et al., 1999). The olefinic sesquiterpenoids isolated from *A. annua* represent different structural classes and a number of sesquiterpene synthases catalyzing the formation of the various carbon skeletons must be expressed in the plant.

Searches by us and other groups have revealed a number of cDNAs from *A. annua* that encode different sesquiterpene synthases (Fig. 1). The first cDNA clone encoding a sesquiterpene synthase from *A. annua* reported was that for *epi*-cedrol synthase (Mercke et al.,

Abbreviations: β-FS, β-farnesene synthase; FPP, farnesyl diphosphate; GPP, geranyl diphosphate; IMAC, immobilized metal affinity chromatography; IPTG, isopropyl thio-β-*D*-thiogalactoside.

☆ The sequence reported in this paper has been deposited in the GenBank Accession No. AY835398.

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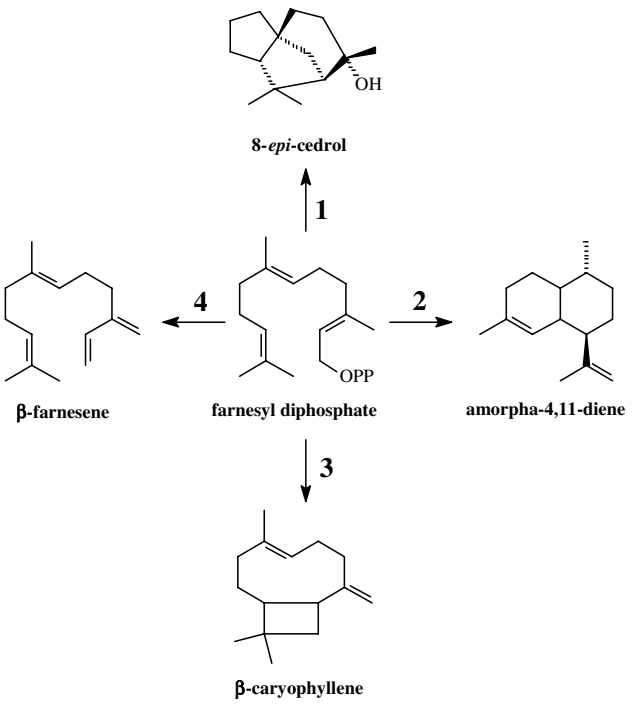

Fig. 1. Reactions catalyzed by the four sesquiterpene synthases cloned from *Artemisia annua*: 1, *epi*-cedrol synthase (ECS); 2, amorpha-4,11-diene synthase (ADS); 3, β-caryophyllene synthase (CS); 4, (*E*)-β-farnesene synthase (FS).

1999; Hua and Matsuda, 1999). A number of groups have reported the molecular cloning of amorpha-4,11-diene synthase (Mercke et al., 2000; Chang et al., 2000; Wallaart et al., 2001). This enzyme is responsible for catalyzing the committed step in the biosynthesis of artemisinin (Bouwmeester et al., 1999). A third cDNA encoding β-caryophyllene synthase has been described (Cai et al., 2002). Van Geldre et al. (2000) reported on the cloning of two putative sesquiterpene synthases from *A. annua*. One of these clones (GenBank Accession No. AJ271972) corresponds to the β-caryophyllene synthase (Cai et al., 2002) while the product selectivity of the other clone (GenBank Accession No. AJ271973) has remained unknown. We have now recloned this sesquiterpene synthase (GenBank Accession No. AY835398) and report that it encodes (*E*)-β-farnesene synthase (β-FS), which converts farnesyl diphosphate to the acyclic sesquiterpene (*E*)-β-farnesene (Fig. 1). (*E*)-β-Farnesene occurs in a wide range of both plant and animal taxa. It is extensively used as a semiochemical in insects and plants. During recent years cDNA clones encoding (*E*)-β-FS has been isolated from peppermint (*Mentha* × *piperita*) (Crock et al., 1997), *Citrus junos* (Maruyama et al., 2001) and *Zea mays* (Schnee et al., 2002). The enzymes from peppermint and *C. junos* produce essentially one product from FPP while the maize enzyme produced three sesquiterpenoids: (*E*)-β-farnesene (26%), (*E*)-β-nerolidol (29%) and (*E*,*E*)-farnesol (45%). (*E*)-β-FS cDNA clone(s) may, by transgenic modification, provide a valuable addition to the arsenal of natural compounds active in host plant resistance.

2. Results and discussion

2.1. cDNA isolation and sequence analysis

Based on the sequence of a putative sesquiterpene synthase from *A. annua* (van Geldre et al., 2000), a 1.8-kb fragment was amplified by PCR and cloned. The full-length clone contained 1746 bp and an open reading frame of 574 amino acids. The deduced mass of the encoded protein is 66.9 kDa and the p*I* is calculated to be 5.03. The low p*I* and the molecular weight are similar to those reported for other plant sesquiterpene synthases. The isolated clone (GenBank Accession No. AY835398) encodes (*E*)-β-FS as determined below.

A comparison of the nucleotide sequence obtained with that of the putative sesquiterpene synthase (GenBank Accession No. AJ271793) (van Geldre et al., 2000) revealed some differences. The deduced amino acid sequences of the two sequences differ in the overall length by three amino acids. Alignment of the two deduced amino acid sequences to those of the other three sesquiterpene synthases from *A. annua* shows that the shorter sequence obtained in this study appears to be correct due to better alignment and higher homology. From this alignment it is also evident that the β-FS has an extension in the N-terminal as compared to the other *Artemisia* sesquiterpene synthases. This extra sequence of 22 residues contains a number of hydroxylated amino acids and it is evident that this extra sequence is highly hydrophilic. According to an iPSORT prediction (http://biocaml.org/ipsort/iPSORT/) this extra sequence does not carry a signal or sorting sequence (Bannai et al., 2002).

The deduced amino acid sequence of β-FS from *A. annua* shows relatively high identity/similarity to other sesquiterpene synthases and especially to those from *A. annua*. It is interesting to note that the β-FS from *A. annua* exhibits a considerably higher homology to other sesquiterpene synthases from this plant (the four *Artemisia* sesquiterpene synthases show an identity/similarity between 47/65% and 59/78% to each other) than to β-FSs from other plants (the *Artemisia* FS shows an identity/similarity of 42/61%, 33/56% and 21/39% to the β-FSs from *Citrus junos*, *Mentha* × *piperita* and *Z. mays*, respectively). Obviously, the level of homology between deduced amino acid sequences of synthases showing identical substrate/product specificity (in this case β-FSs) from different species can be lower than the homology between synthases catalyzing the formation of completely different terpene structural types from the same plant.

2.2. Bacterial expression

Production of recombinant protein was carried out under standard conditions using *Escherichia coli* BL21(DE3) cells transformed with the bacterial expression vector pET28 carrying the β-FS cDNA. SDS–PAGE revealed an IPTG inducible band at around 65 kDa. The apparent molecular mass is somewhat smaller than the calculated size (69.1) for the fusion protein composed of the β-FS and the histidine leader peptide derived from pET28.

Crude extracts of *E. coli* expressing the cloned sesquiterpene synthase displayed a high activity with [1-^{3}H]-FPP as substrate. GC–MS analysis of the enzymatic product revealed the formation of a single sesquiterpenoid (peak 2 in Fig. 2(a)). Comparison of the MS-spectrum of this product (Fig. 2(b)) to spectra of the parent compound reveals that the enzyme catalyzes the formation of (*E*)-β-farnesene (Fig. 2(c)).

2.3. Purification of recombinant β-farnesene synthase

In order to facilitate an easy purification of the β-FS, the enzyme was expressed in fusion with a N-terminal affinity tag using the bacterial expression vector pET28. This vector encodes a hexa-histidyl leader sequence under the control of the T7 RNA polymerase promoter. The enzyme is extended with 20 amino acids after fusion to the His_6-tag.

Recombinant β-FS was purified by immobilized metal affinity chromatography (IMAC) on a HiTrap Chelating HP column loaded with Co^{2+}. From SDS–PAGE it is evident that a relatively efficient purification of the recombinant enzyme is achieved after the IMAC (data not shown). Western blotting experiments using rabbit polyclonal amorpha-4,11-diene synthase antibodies showed that the purified β-FS cross-reacts with these antibodies (data not shown).

2.4. pH-Optimum of β-farnesene synthase

The recombinant purified β-FS exhibited maximum activity at around pH 6.5 to 7.0 (data not shown). Many plant sesquiterpene synthases exhibit maximum activity in the pH range of 6.5–7.5. Plant sesquiterpene synthases are generally fully active at neutral pH values. This is in line with the assumption that they are localized to the cytosol of the cell.

2.5. Divalent metal ion dependence of β-farnesene synthase

Sesquiterpene synthases have an absolute requirement for a divalent metal ion as cofactor. The activity of β-FS has been measured in the presence of a number of different divalent metal ions (Fig. 3(a)). Substantial

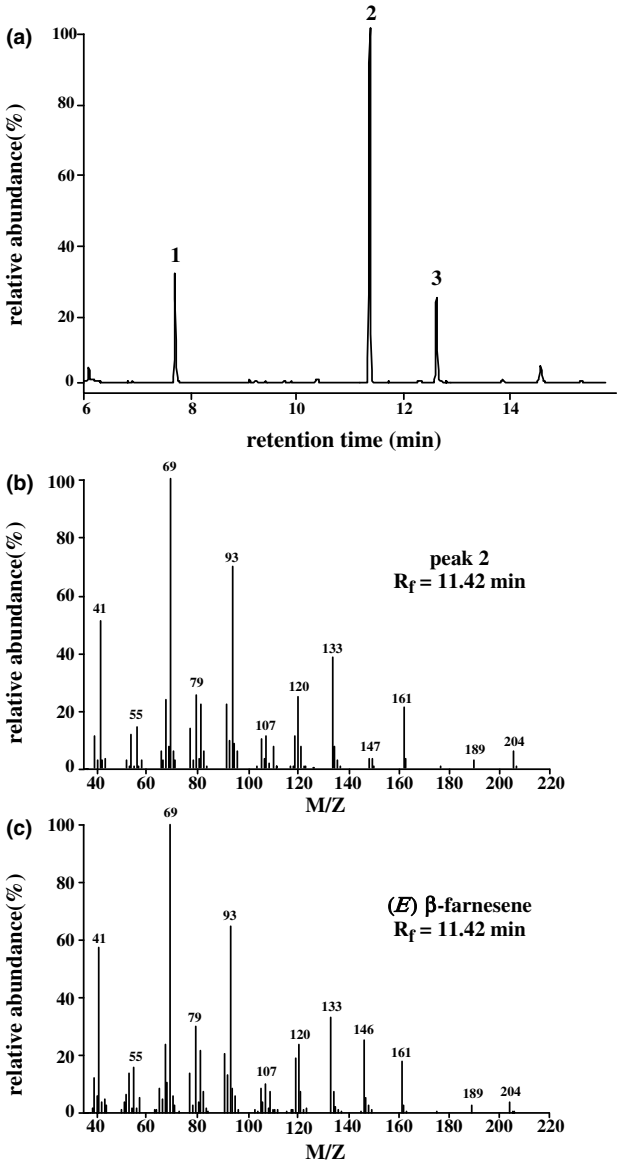

Fig. 2. GC–MS analysis of the sesquiterpene product of the recombinant enzyme: (a) GC elution profile of the olefinic products generated by from FPP by the recombinant enzyme. Peak 1 and peak 3 are not sesquiterpenoids; (b) mass spectrum of the major biosynthetic product (R_f = 11.42) (peak 2); (c) mass spectrum of *trans*-β-farnesene (R_f = 11.42).

activity is observed in the presence of Mg^{2+} or Co^{2+}. In the presence of Mn^{2+}, Ni^{2+}, Cu^{2+} or Zn^{2+} essentially no activity is observed at the tested concentrations. However, the β-FS activity is highly dependent on the concentration of the ion used and at low concentrations of Mn^{2+} the β-FS exhibits a relatively high activity (Fig. 3(d)). From Fig. 3(b) to (d), it is clear that maximum β-FS activity is observed at different metal ion concentrations. The concentration required for maximum activity are estimated to 5 mM (Fig. 3(b)), 0.5 mM (Fig. 3(c)) and <10 μM (Fig. 3(d)) for Mg^{2+}, Co^{2+} or Mn^{2+}, respectively. However, using Mn^{2+} or Co^{2+} as cofactor a

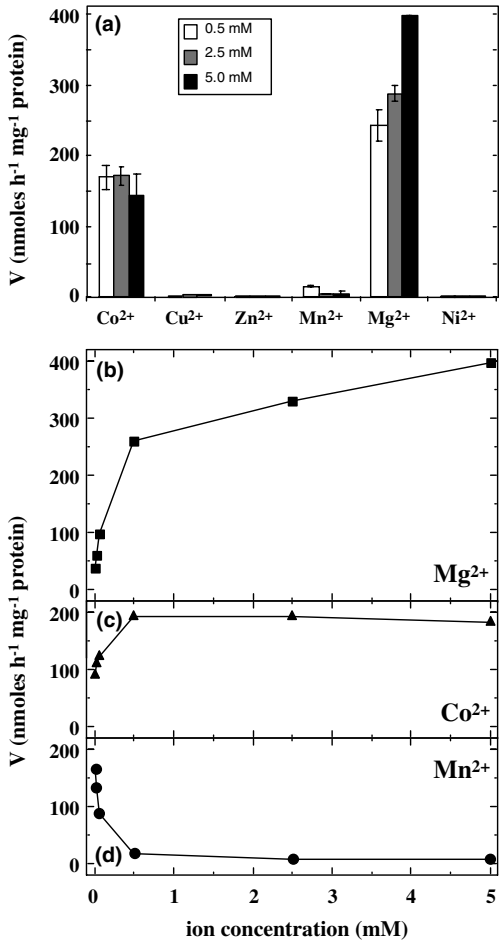

Fig. 3. Activity of β-FS in the presence of different divalent metal ions: (a) β-FS activity at three different concentrations of divalent metal ions; (b) β-FS activity as function of Mg^{2+} concentration; (c) β-FS activity as function of Co^{2+} concentration; (d) β-FS activity as function of Mn^{2+} concentration.

somewhat lower specific β-FS activity is observed than in the presence of Mg^{2+}. In addition to this, the enzyme is strongly inhibited by increased concentrations of Mn^{2+} at a relatively low concentration (>20 μM). Inhibition by high Mn^{2+}-concentrations has also been observed for amorpha-4,11-diene synthase (Picaud et al., 2004), and (*E*)-nerolidol synthase from maize (Degenhardt and Gershenzon, 2000). In a number of studies, it has been shown that maximum enzyme activity is reached at a considerable lower concentration of Mn^{2+} than Mg^{2+} for sesquiterpene synthases (Cai et al., 2002; Schnee et al., 2002; Picaud et al., 2004) but in general the specific activity is lower with Mn^{2+} as cofactor.

2.6. Steady-state kinetics of β-farnesene synthase

Steady-state kinetic parameters of the purified recombinant (*E*)-β-FS were determined at pH 7.0. The K_m-value (2.1 μM), k_{cat}-value (9.5×10^{-3} s^{-1}) and enzyme efficiency ($k_{cat}/K_m = 4.5 \times 10^{-3}$ M^{-1} s^{-1}) recorded for FPP in the presence of Mg^{2+} is in the same range as those for other plant sesquiterpene synthases. As reflected by the k_{cat}-value, this enzyme is a relatively slow enzyme, which is a general feature of terpene synthases. Sesquiterpene synthases exhibit turnover numbers in the range 0.01–0.3 s^{-1} (Cane, 1990). Pre-steady-state kinetic analysis on tobacco *epi*-aristolochene synthase (Mathis et al., 1997) and trichodiene synthase (Cane et al., 1997) implies that plant sesquiterpene synthases operate by a rapid equilibration with FPP to form a precatalytic enzyme–substrate complex. The next step is a slightly slower conversion of FPP to hydrocarbons. The release of products is most likely the rate-limiting step of the reaction.

2.7. Substrate specificity of β-farnesene synthase

In the presence of Mn^{2+} but not with Mg^{2+}, GPP is a substrate for different sesquiterpene synthases (Cai et al., 2002; Colby et al., 1998; Köllner et al., 2004; Mercke et al., 1999; Picaud et al., 2004). However, the purified recombinant (*E*)-β-FS from *A. annua* does not convert GPP to product in the presence of Mg^{2+}- or Mn^{2+}-ions at any of the concentrations tested (10 μM to 5 mM).

2.8. Products formed by β-farnesene synthase

The recombinant (*E*)-β-FS produces a single sesquiterpene during catalysis as illustrated in Fig. 2(a). Many recombinant sesquiterpene synthases give rise to more than one product (Crock et al., 1997; Colby et al., 1998; Mercke et al., 1999; Picaud et al., 2004; Steele et al., 1998). Most often one major product and a number of by-products are obtained. Premature or alternative quenching of the reaction cascade may cause the formation of by-products. Other α- and β-farnesene synthases also show high product selectivity (Crock et al., 1997; Martin et al., 2004; Mercke et al., 2004; Pechous and Whitaker, 2004).

3. Experimental

3.1. Reagents

Restrictions enzymes *Spe*I, *Nhe*I and *Sac*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. DNA BigDye™ Terminator Cycle Sequencing Kit was obtained from Perkin–Elmer. HiTrap Chelating HP and PD-10 columns Sephadex™ G-25M were from Amersham-Pharmacia Biotech. [1-^{3}H]-FPP (0.59 TBq/mmol) was purchased from Amersham-Pharmacia Biotech and [1-^{3}H]-GPP (0.56 Tbq/mmol) from American Radiochemical Co. IPTG was obtained from Saveen Werner

AB. FPP, GPP and kanamycin were obtained from Sigma. All other biochemicals and reagents were purchased from commercial sources.

3.2. Plant growth

Plants of *A. annua* were grown from seeds in a green house at 20 °C with a 16-h light period.

3.3. Cloning of the β-farnesene synthase cDNA by RT-PCR

RNA from mature *A. annua* leaves harvested at flowering stage was extracted with the Purescript® Total RNA Isolation Kit (Gentra Systems) according to the manual. First strand cDNA synthesis was initiated on 1.0 μg of total RNA at the poly(A)-tail of mRNA using the adapter primer (AP) and the Superscript II reverse transcriptase (Invitrogen). After synthesis of the first cDNA strand, mRNA was destroyed with RNase H (Invitrogen). The complete open reading frame was amplified by PCR using the cDNA synthesized as template and using specific primers designed from the sequence putative sesquiterpene synthase (GenBank Accession No. AJ271793) (van Geldre et al., 2000). The primers used were: for the forward primer 5′-gatactagtatgtcgactcttcctatttctag-3′ including the restriction site *Spe*I (underlined) and the reverse primer 5′-cagagagctcttagacaaccatagggtgaac-3′ including the restriction site *Sac*I (underlined). The obtained DNA fragment with a length of 1746 bp was electrophoresed through a 1% agarose gel. After gel purification, the PCR fragment was A-tailed using the Taq-polymerase (Invitrogen) and cloned into the plasmid vector pGEMT-Easy (Promega). The plasmid obtained was transformed into competent *E. 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 using the BigDye™ Terminator Cycle Sequencing Kit for labeling of the sequencing reactions. Collection of the sequence data was carried out on an ABI PRISM™ 310 Genetic Analyzer. Oligonucleotides were synthesized according to the sequence information and used directly as primers for further sequencing. The cDNA fragment was excised from pGEMT-Easy vector by restriction digestion with *Spe*I and *Sac*I and transferred into the pET28 expression vector (Novagen) digested by *Nhe*I and *Sac*I in frame with an affinity tag (His_6). Transformants were selected on LB plates containing kanamycin (50 μg/ml).

3.4. Bacterial expression of the β-farnesene synthase

The plasmid pET28 containing the cDNA fragment was transformed into competent *E. coli* BL21(DE3) cells according to standard procedures. A single colony was used to inoculate 10 ml LB medium containing kanamycin (50 μg/ml) at 37 °C overnight. Two ml of this culture were added to 200 ml of fresh LB containing kanamycin. Bacteria were grown at 37 °C to $OD_{600} = 0.5$. Induction was achieved by addition of 0.2 mM IPTG. The cells were maintained for 4 h at 30 °C and then harvested by centrifugation (2000*g*, 10 min). Subsequently, the bacteria were suspended in half of the previous volume in 20 mM sodium phosphate buffer pH 6.5 containing 10% (v/v) glycerol and 0.5 M NaCl. After a new centrifugation and re-suspension of the cells in 1/10 of the original culture volume, sonication of the cells was carried out for 2 min in 5 s pulses with 5 s between pulses on ice (Vibracell power sonicate setting 12– 15 W). The cell lysate was then centrifuged at 10000*g* for 30 min at 4 °C and the supernatant, containing the soluble recombinant enzyme was filtrated on a 0.45 μm non-pyrogenic sterile filter.

3.5. Purification of β-farnesene synthase

The cell lysate containing the soluble recombinant enzyme was passed through a 5 ml HiTrap Chelating HP column (Amersham Biosciences) loaded with Co^{2+}. After washing with 30 mM imidazole, bound enzyme was eluted in 20 mM sodium phosphate buffer, pH 6.5, containing 10% (v/v) glycerol, 0.5 M NaCl and 300 mM imidazole. Fractions (1 ml) were collected and fractions containing activity were pooled and passed through a PD-10 column (Amersham Biosciences) equilibrated with 20 mM sodium phosphate buffer, pH 6.5, containing 10% (v/v) glycerol and 0.5 M NaCl to remove imidazole. The purified enzyme was frozen in aliquotes in liquid nitrogen and store at −20 °C until used.

3.6. Electrophoresis and western blotting

SDS–PAGE electrophoresis was carried out using MES-buffer on 4–20% Tris/glycine gels or on NuPAGE 4–12% Bis/Tris gels from Invitrogen according to instructions supplied by the manufacturer using a Novex mini electrophoresis cell. After electrophoresis, the gels were either stained with Coomassie blue or the proteins transferred to nitrocellulose membranes using a Mini Trans-Blot module (Novex) according to instructions supplied by the manufacturer. The correct transfer was checked by including a mixture of prestained protein markers on the PAGE gel (Kaleidoscope Prestained Standards; Bio-Rad). Rabbit polyclonal ADS antibodies and goat-anti-rabbit conjugated with alkaline phosphatase were use as primary and secondary antibodies, respectively. The blot was developed with the 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium system according to the manufacturer's instructions (Bio-Rad).

3.7. Standard enzyme assay

An aliquot of bacterial extract (48 μl) or purified enzyme (10 μl) was assayed in 20 mM sodium phosphate buffer, pH 6.5, containing 5 mM $MgCl_2$, 10% glycerol and 23 μM [1-^{3}H]-FPP (1.4 MBq) in a total volume of 50 μl. After 20 min incubation at 30 °C, the reactions were stopped by addition of an equal volume of 0.2 M KOH containing 0.1 M EDTA. Subsequently, the reaction mixture was extracted with hexane (0.5 ml) and the hexane extracts were passed over a small column filled with 250 mg of silica (Merck; size: 0.2–0.5 μm) into a scintillation vial. The column was rinsed with additional hexane (2 × 1 ml), which was collected in the same scintillation vial. Vials were examined for radioactivity by scintillation counting on TRI-CARB 2100 TR Liquid scintillation Analyser (Packard). Three replications were run for each sample.

3.8. pH optimum determination

For characterization, the purified recombinant enzyme was used (see above). The pH optimum was determined in buffers of 50 mM Bis–Tris, 50 mM sodium phosphate or 50 mM Tris–HCl containing 5 mM $MgCl_2$ and 10% glycerol, in the pH range from 5.0 to 9.5 using 1.5 μg of the purified protein and 18.7 μM [1-^{3}H]-FPP in a final volume of 100 μl. Samples were incubated at 30 °C for 10 min.

3.9. Kinetic analysis of the β-farnesene synthase

Kinetic analysis of enzymes was conducting using 1.5 μg of purified protein per assay (100 μl). The assays were conducted according to the standard assay with 0.5, 1, 2, 5 or 10 μM [1-^{3}H]-FPP. K_m-constants were determined from Lineweaver–Burk plots and k_{cat}-values were estimated from the protein concentration using a molecular weight of 69.1 kDa (β-FS + affinity tag).

3.10. Effects of cation concentration on enzyme activity

The effects of divalent metal ions (Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+} or Cu^{2+}) on enzyme activity were initially measured at 3 different ions concentrations, i.e., 0.5, 2.5 and 5.0 mM. Then the saturation concentration of Mg^{2+}, Mn^{2+} or Co^{2+} was determined by varying the concentration of the ion in the standard assay in the range from 10 μM to 5 mM.

3.11. Product analysis

The purified protein (4 × 8 ml containing 150 μg protein each) were supplemented with 5 mM $MgCl_2$ and 23 μM FPP. The mixture was incubated in glass tubes at 30 °C for 5 h. Then the mixtures were extracted twice with equal volumes of *n*-pentane (Sigma). The 4 fractions obtained were pooled and the pentane phase was concentrated by evaporation under a stream of helium gas to around 50 μl. The sample was analyzed by capillary GC on an Agilent 6890 gas chromatograph equipped with a HP-5M5 5% phenyl methyl silohexane capillary column (0.25 μm film thickness, 0.25 mm i.d. × 30 m) and an Agilent 5973 Network Mass Selective Detector. The GC injection port was operated at 100 °C. The oven temperature was programmed from 100 to 200 °C at 5 °C min^{-1} and from 200 to 250 °C at the rate of 25 °C min^{-1}. The final temperature was maintained for 26 min. Helium was used as a carrier gas and sample (1.0 μl) was injected in split mode. Mass spectra were recorded in electron impact mode at 70 eV with scanning from *m*/*z* 34 to 400 at 3.93 scans s^{-1}. Sesquiterpenoids were identified by comparing the mass spectra and retention indices with those of spectra found in the Mass Finder 2.1 database and other libraries.

3.12. Protein concentrations

Protein concentrations of extracts and purified recombinant β-FS were determined according to Bradford (1976) using IgG as standard.

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