Biochemical and Biophysical Research Communications 458 (2015) 883–889

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

SUMO-fusion, purification, and characterization of a (+)-zizaene synthase from *Chrysopogon zizanioides*

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ARTICLE INFO

Article history:
Received 6 February 2015
Available online 19 February 2015

Keywords:
Terpene synthase
Sesquiterpenes
Zizaene
Khusimene
Enzyme kinetics
SUMO

ABSTRACT

An uncharacterized plant cDNA coding for a polypeptide presumably having sesquiterpene synthase activity, was expressed in soluble and active form. Two expression strategies were evaluated in *Escherichia coli*. The enzyme was fused to a highly soluble SUMO domain, in addition to being produced in an unfused form by a cold-shock expression system. Yields up to ~325 mg/L^{-1} were achieved in batch cultivations. The 6x-His-tagged enzyme was purified employing an Ni^{2+}-IMAC-based procedure. Identity of the protein was established by Western Blot analysis as well as peptide mass fingerprinting. A molecular mass of 64 kDa and an isoelectric point of pI 4.95 were determined by 2D gel electrophoresis. Cleavage of the fusion domain was possible by digestion with specific SUMO protease. The synthase was active in Mg^{2+} containing buffer and catalyzed the production of (+)-zizaene (syn. khusimene), a precursor of khusimol, from farnesyl diphosphate. Product identity was confirmed by GC–MS and comparison of retention indices. Enzyme kinetics were determined by measuring initial reaction rates for the product, using varying substrate concentrations. By assuming a Michaelis–Menten model, kinetic parameters of $K_M = 1.111$ μM (±0.113), $v_{max} = 0.3245$ μM min^{-1} (±0.0035), $k_{cat} = 2.95$ min^{-1}, as well as a catalytic efficiency $k_{cat}/K_M = 4.43 \times 10^4$ M^{-1} s^{-1} were calculated.

Fusion to a SUMO moiety can substantially increase soluble expression levels of certain hard to express terpene synthases in *E. coli*. The kinetic data determined for the recombinant synthase are comparable to other described plant sesquiterpene synthases and in the typical range of enzymes belonging to the secondary metabolism. This leaves potential for optimizing catalytic parameters through methods like directed evolution.

1. Introduction

Sesquiterpenes and their derivative sesquiterpenoids are essential fragrance components in deodorant, shower gel or perfume and thus used (and smelled) by us on a daily basis. These molecules constitute a diverse class of natural compounds, produced enzymatically in both eukaryotes and prokaryotes as secondary metabolites. Especially plants utilize these olfactory active C_{15} hydrocarbons either to attract beneficial insects (pheromones), or to defend predators [1]. Sesquiterpenes are cyclization products of the universal precursor farnesyl diphosphate (FDP). The substrate, bound to a sesquiterpene synthase (STPS), undergoes cleavage of the pyrophosphate in the active site, resulting in various C–C bonding reactions, cyclizations, and subsequent hydrid shifts. Whether STPSs actively control and direct those reactions, or barely serve as structural and stereochemical templates forming the molecule, is still not fully understood [2].

STPSs show a broad sequence and structural variability [3]. Nevertheless, there are some similarities present among this enzyme class. Plant STPS cDNAs are usually around 1500–1700 nucleotides in length, resulting in a translated protein of roughly 60–70 kDa molecular weight (not considering possible glycosylation *in planta*). They also share two common sequence motifs, dubbed the DDXXD and NSE/DTE motifs. These aspartate rich regions are known to be involved in binding a trinuclear magnesium cluster [4]. Magnesium or related metal cations as manganese are essential for enzymatic activity. No further cofactors are needed. Temperature optima are mainly in the typical physiological range of 20–40 °C.

Abbreviations used: STPS, sesquiterpene synthase; FDP, farnesyl diphosphate; SUMO, small ubiquitin-related modifier; IMAC, immobilized metal ion affinity chromatography.

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http://dx.doi.org/10.1016/j.bbrc.2015.02.053
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Due to their mostly transient expression in plants as well as their low concentration in the relevant tissue, it is still considered challenging to identify, sequence, and characterize these enzymes [7]. The fraction of sequenced and characterized plant sesquiterpene synthases today is disproportionate, considering the vast number of synthases suspected to be available in nature. The Brenda Enzyme database [8] lists 68 different STPSs (as of 2014) with EC numbers in the subgroup 4.2.3.X (carbon–oxygen lyases acting on phosphates) and which bind FDP as a substrate. Not all of those have been intensively characterized and described, both in respect to expression in heterologous organisms as well as enzyme kinetics. X-ray crystal structures have been deposited for less than a dozen unique plant synthases as of today [9,10]. Not much information is available concerning the exact mechanisms of building the hydrocarbon terpene skeleton, especially when many different products are formed by the synthase [11,12].

Khusimol can be considered as one interesting and important fragrance molecule, due to its pleasant olfactory properties. Next to α- and β-vetivone, khusimol is the main component in the woody-smelling essential oil of *Chrysopogon zizanoides* (syn. *Vetiveria zizanoides*), also known as vetiver grass [13]. Khusimol is extracted from the exotic grass, along with the essential oil, by distillation of harvested roots [14]. It would be possible to derive khusimol (Fig. 1) by site-specific chemical or enzymatic oxidation of the methyl group of a precursor called zizaene (syn. khusimene). It was shown earlier, that a stereocontrolled total synthesis of (+)-zizaene is possible in lab scale [15,16], nevertheless not feasible for production due to very low yields and many steps involved. A sustainable route based on biocatalytic transformation of this precursor seems practical, as a STPS can form complex sesquiterpenes in a one-step, enantioselective reaction.

The unannotated nucleotide sequence GenBank: HI931360 coding for a polypeptide presumably having zizaene synthase activity was published previously [17], although no further expression studies, purification protocols as well as characterization of key enzymatic properties were described to date.

2. Materials & methods

2.1. Cloning of expression constructs

The unannotated cDNA sequence GenBank: HI931360 [17] was carefully codon-optimized for expression in *Escherichia coli* K12 derivative strains using a guided-random approach [18]. The optimized sequence (1668 bp) GenBank: KP231534 was ordered as two individual double stranded DNA fragments of equal length using the GeneArt© Strings™ service (Life Technologies, USA). All constructs were cloned by use of the proprietary Seamless cloning & assembly system (Life Technologies, USA), a method related to other PCR-based cloning methods e.g. Gibson assembly [19]. For every plasmid construct, six primers were designed to create a 15 bp overlap of the inserts and vectors (Table S1). The circular vectors pET16b, pETSUMO (both Life Technologies, USA), pColdI (TaKaRa Bio Europe/SAS, France), as well as both synthetic DNA strings were amplified using Q5® High-Fidelity DNA Polymerase (NEB, USA) to create long and error-free linear fragments. Typical PCR reactions were conducted as follows: 10 µl 5× Q5 HF buffer, 1 µl dNTP mix (10 mM each), 1 µl DNA template, and 0.5 µl Q5 DNA polymerase in a total volume of 50 µl (add H_2O). Standard PCR cycler program for insert strings: initial denaturation at 98 °C for 30 s; 15 cycles at 98 °C for 10 s (denaturation), 72–62 °C (Touchdown PCR annealing [20]) for 30 s, and 72 °C for 30 s. Additional 20 cycles at 62 °C. The PCR reactions with plasmid DNA as template were digested with DpnI (NEB, USA) for 1 h at 37 °C to decrease background after transformation. PCR fragments were purified using QIAquick PCR Purification Kit (QIAGEN GmbH, Germany), analyzed in analytical agarose gels, and used in the assembly reaction. Competent *E. coli* TOP10 cells (Life Technologies, USA) were chemically transformed with 8 µl of the fused construct. Clones were screened for correct plasmid by colony PCR with gene and vector specific primers. Plasmids were purified following common protocols, sequenced, and chemically transformed into *E. coli* BL21(DE3) (Merck Millipore, Germany) for expression studies. Annotated plasmid maps are available (Figs. S1–3).

2.2. Expression experiments in E. coli

Cultivations were performed in complex TB (terrific broth) medium (12 g/L tryptone, 24 g/L yeast extract, 4 ml/L glycerol, 100 ml/L 10× potassium phosphate buffer) supplemented with the respective antibiotics for plasmid stability (pET16b::ZIZ(co) and pColdI::ZIZ(co) 100 µg/ml carbenicillin, pETSUMO::ZIZ(co) 50 µg/ml kanamycin). Pre-cultures were inoculated directly from cryostocks and grown 8 h over-night before being diluted to an initial optical density of OD_{600} 0.05 rel. AU in the main cultures. After growth for ~4 h at 37 °C at 180 rpm (OD_{600} 0.8–1 rel. AU), culture vessels were rapidly cooled in ice-water and induced by IPTG (conc. 0.1 mM–1 mM). Cultivation was continued for up to 24 h at 20 °C, 180 rpm (15 °C, 180 rpm in case of pColdI::ZIZ(co) harboring strains). Samples were normalized according to the optical density, harvested (14,000 × g, 4 °C, 15 min), resuspended in sesquiterpene activity buffer (50 mM MOPS pH 7.5, 1 mM DTT, 10 mM $MgCl_2$, 10% (v/v) glycerol), and sonicated 10 times 20 s on ice (Sartorius Labsonic M, Germany) using parameters: 0.6 s cycle, 100% amplitude. After clearing the lysate (14,000 × g, 4 °C, 60 min), the soluble supernatant was directly used in SDS-PAGE analysis. Insoluble

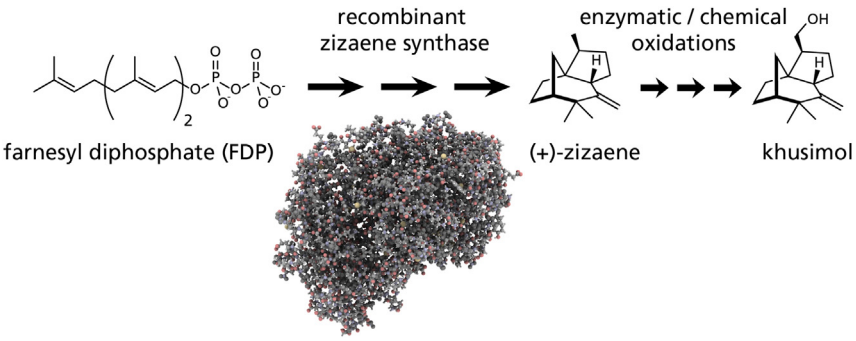

Fig. 1. Reaction catalyzed by the recombinant (+)-zizaene synthase discussed in this study. FDP is cyclized to form (+)-zizaene, which is a direct precursor to the oxidized derivative compound khusimol. Putative structure of recombinant zizaene synthase modeled from aa sequence using I-Tasser algorithm [5] and rendered in QuteMol [6].

protein pellets were resuspended in denaturing resuspension buffer (100 mM potassium phosphate pH 7.0, 10 mM sodium metabisulfite, 10 mM β-mercaptoethanol, 10 mM ascorbic acid, 6 M urea) and solubilized by vigorous shaking at 22 °C for 1 h before being analyzed. PageRuler Prestained Protein Ladder #26616 and Pierce Unstained Protein MW Marker #26610 (both Thermo Scientific, USA) were loaded as molecular weight markers. PAGE gels were stained by colloidal coomassie staining [21]. Western Blots were conducted after semi-dry plotting on PVDF membrane, using 6x-His epitope tag mouse antibody #MA1-21315 (Thermo Scientific, USA) as primary and goat anti-mouse HRP conjugate #401215 (Calbiochem, USA) as secondary antibody. Blots were stained using 3,3′,5,5′-Tetramethylbenzidine (TMB) as substrate.

2.3. Purification of the recombinant sesquiterpene synthase

Induced biomass was harvested after 24 h, resuspended in IMAC binding buffer (50 mM MOPS pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 50 mM imidazole) to yield a concentration of 100 mg wet cell-mass/ml. Purification was performed on a FPLC system BioLogic DuoFlow (Bio-Rad Laboratories Inc., USA) equipped with a HiTrap™ IMAC FF 5 ml column (GE Healthcare, USA), decorated with Ni^{2+} cations. Samples were prefiltered using 0.2 μm syringe filters. After binding to the ligand, unbound protein was washed from the column before eluting the target fractions with IMAC elution buffer (binding buffer, except 500 mM imidazole). Product fractions were pooled, concentrated using Vivaspin 20 ultrafiltration modules with a MWCO of 10 kDa (Sartorius-Stedim Biotech, Germany). Buffer was exchanged to aforementioned sesquiterpene activity buffer in three subsequent ultrafiltration steps. Aliquots (100 μl) were snap-frozen in liquid N_2, and stored at −80 °C.

2.4. Biotransformation assays and GC-FID/GC–MS analytics

A single-vial assay method for bioactivity testing of sesquiterpene synthases [22] was adapted. Purified enzyme was diluted to 25 μg/ml in a final volume of 1 ml activity buffer previously supplemented with 150 μM (final concentration) FDP tris-ammonium salt (Mobitec, Germany). Reaction mixtures were promptly overlaid with 200 μl iso-octane, incubated for 15 min at 30 °C, extracted by vigorous shaking, and the organic phase analyzed by GC-FID.

Sesquiterpenoid enzyme products were detected by a Shimadzu GC-2001 plus gas chromatograph equipped with a Phenomenex Zebron ZB-Wax Plus column (30 m length, I.D. 0.25 mm, 0.25 μm film thickness). Organic phases (2 μl) were injected via autosampler (split-less mode, split temperature 240 °C). Temperature gradient was as follows: 40 °C hold for 20 s, raise to 200 °C (10 °C/min) and hold for 0.5 min, raise to 230 °C (30 °C/min) and hold for 2 min. The FID was heated to 300 °C for detection of molecule fragment ions.

For initial identification of sesquiterpenoid compounds, GC–MS was performed on a Fisons GC 8000 gas chromatograph connected to a Fisons MD 800 mass selective detector (interface 230 °C; ion source 200 °C; quadropole 100 °C; EI ionization 70 eV; scan range 33–300 amu; cool on-column injection). Helium was used as carrier gas (38 cm/s) on a Phenomenex Zebron ZB-WAX column (parameters as mentioned above). Mass spectra were compared to a digital library (Wiley08/NIST08, 2008) and manually matched to published spectra of authentic standards [28].

2.5. Determination of enzyme kinetics

The recombinant enzyme product was shown to be apparently homogenous by SDS-PAGE and Western Blots after purification. Enzyme concentration was determined by densitometry of coomassie-stained SDS-PAGE gels using BSA (bovine serum albumin) as standard (ImageJ software [23]). In addition, the enzyme was quantified by measuring adsorption at 280 nm (theoretical MW 65.98 kDa, extinction coefficient 88,210 M^{-1} cm^{-1}) with Nano-Drop 1000 UV–Vis spectrophotometer (Thermo Scientific, USA). Enzyme kinetic parameters for the purified, His-tagged zizaene synthase were determined by adapting a discontinuous kinetic assay with direct determination of product concentrations via GC-FID. The underlying reaction equation was assumed to be (2E,6E)-farnesyl diphosphate→ (+)-zizaene + diphosphate. In preliminary tests, an enzyme concentration of ~0.1 μM was found to be suitable for the initial reaction slope to be in the linear range. The addition of the metal cofactor Mg^{2+} was essential for activity of the enzyme. For each assay run, a master mix consisting of sesquiterpene activity buffer (50 mM MOPS pH 7.5, 1 mM DTT, 10 mM $MgCl_2$, 10% (v/v) glycerol) and the substrate (2E,6E)-FDP (1 g/L stock solution, MW 433.42 g/mol) in varying concentrations (0 μM, 1 μM, 5 μM, 10 μM, 20 μM, 30 μM, 40 μM) was prepared. A master mix was rapidly split in five individual reaction tubes (500 μl each), immediately overlaid with 200 μl iso-octane, and promptly transferred to a preheated water bath (30 °C). Tubes were taken from the water bath in 2 min intervals from 0 to 10 min after addition of the enzyme. The reaction was stopped by vigorously shaking the reaction mixture for 30 s, a short centrifugation step for phase separation, and immediate transfer of the upper organic phase in a GC vial for quantification. The initial reaction slope for each run was determined by linear regression and plotted against the initial substrate concentration. Fitting the data to the Michaelis–Menten kinetic model was conducted using Origin (OriginLab, USA).

3. Results and discussion

3.1. Soluble expression in E. coli and purification of the recombinant synthase

The codon adaption index (CAI [24]) for nucleotide sequence HI931360 [17] was raised from 0.551 to 0.707. Protein BLAST search of the translated sequence identified a β-sesquiphellandrene synthase C5YHI2 and a (*E*)-β-caryophyllene synthase ABY79211 with the highest sequence similarity (identity < 52%). Alignment with closely related terpene synthases revealed the conserved DDXXD as well as the NSE/DTE motif to be present in the protein sequence, thus categorizing the peptide in the terpene cyclase superfamily (Figure S4). Two expression strategies were subsequently evaluated. N-terminal fusion to an easily cleavable SUMO moiety, previously shown to enable and increase soluble expression levels for certain proteins [25,26], as well as cold-shock induced expression exploiting the cspA promoter [27]. Both strategies enabled soluble and active expression of the recombinant enzyme, as evaluated by SDS-PAGE analysis (Fig. 2, A + B). Inducer concentration had no significant impact on protein yield.

It is suspected, that the rapid folding of a highly soluble and structurally compact SUMO moiety in *Escherichia coli* serves as nucleation site for folding of a polypeptide attached to its C-terminus [26]. Conversely, in case of the pColdI::ZIZ(co) harboring strain, cold shock induction at 15 °C slowed the translation machinery to increase the timeframe for proper folding of the nascent peptide chain.

Cloning of the artificial cDNA construct in pET16b and expression in shake flask scale yielded no soluble expression even at temperatures below 20 °C using varying IPTG inducer concentrations (Fig. 2, C).

With soluble enzyme yields of up to ~325 mgL^{-1} vs. ~221 mgL^{-1} (densitometric analysis, shake flask scale, TB medium), the SUMO-fusion strategy yielded superior amounts compared to cold-shock

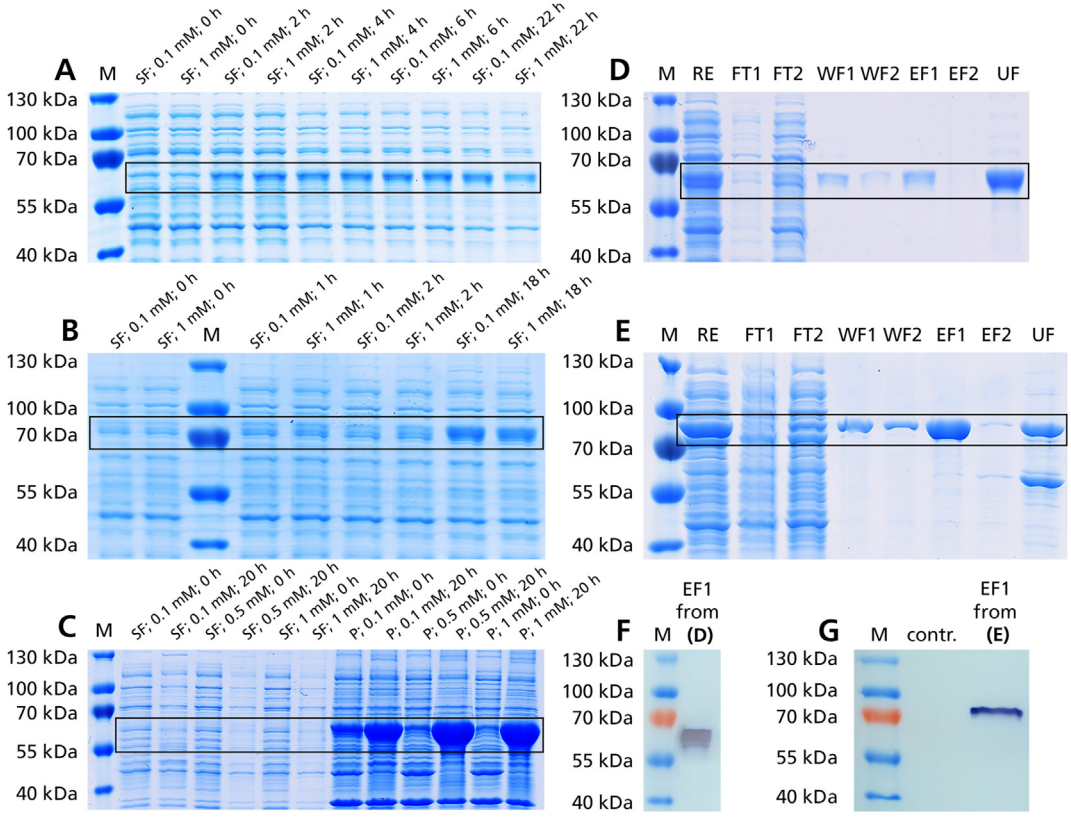

Fig. 2. SDS-PAGE gel scans illustrating expression experiments in complex TB medium of strains harboring pColdI::ZIZ(co) (A), pETSUMO::ZIZ(co) (B), and pET16b::ZIZ(co) constructs (C). Varying IPTG inducer concentrations (depicted in mM) were used. Cultivation temperature of 20 °C after induction (except pColdI-strains). Purification of unfused (+)-zizaene synthase (D) and (+)-zizaene synthase fused to SUMO (E) using IMAC based affinity chromatography. Western-Blots using His-tag epitope specific antibodies of respective elution fractions (F, G). SF = soluble fraction; P = pellet fraction; RE = raw extract; FT = flow through; WF = wash fraction; EF = elution fraction; UF = after ultrafiltration with 10 kDa MWCO.

induced cultures. Both strategies examined are suitable solutions to overcome formation of insoluble protein aggregates.

Purification of the synthase was conducted by binding raw extracts of sonicated biomass to a Ni^{2+}-decorated IMAC sepharose column. Following subsequent wash-steps, elution of the enzyme was achieved by applying an imidazole gradient. Elution fractions were pooled and dialyzed by ultrafiltration against sesquiterpene activity buffer with 10 kDa MWCO (Fig. 2, D + E). Purity of enzyme was >95%, determined by densitometry. Western-Blots with His-epitope antibody (Fig. 2, F + G) as well as analysis of the peptide mass fingerprint (10 peptides matched, 17.5% sequence coverage) verified the identity of the expressed polypeptide (Figure S5).

Cleavage of the SUMO fusion protein was achieved by incubating the purified enzyme with protease ULP1 (Ubl-specific protease 1), cleaving the 13 kDa SUMO moiety from the 64 kDa synthase (Figure S6). Because the 6x-His-tag was attached N-terminally, a subsequent IMAC purification step yields the fusion-protein free enzyme in the flow through fraction.

To determine the isoelectric point, a 2D gel electrophoresis was conducted including an isoelectric focusing step in the range of pH 3–10. The native pI was determined to be pI~4.95, which lies very close to the theoretical calculated value of pI 5.09 for the cleaved synthase (Figure S7).

3.2. Activity assays and identification of enzyme products

After cell lysis and centrifugation, the soluble supernatants of pETSUMO::ZIZ(co) and pColdI::ZIZ(co) BL21(DE3) strains were diluted in sesquiterpene activity buffer and the solution supplemented with 50 μM FDP tris-ammonium salt. GC-FID analysis of the organic iso-octane phase after incubation at 30 °C for 30 min showed the production of one main enzymatic product for both the SUMO-fused and unfused synthase (Fig. 3). No product formation was detectable for the control without plasmid insert (Figure S8). The main peak 1 was further analyzed by GC–MS. The retention index (RI 1613 calculated, RI 1597 in literature for related column [29]), as well as the fragment ion spectrum (main peaks m/z 134, 91, 41, 79, 119 and M^+ 204) matched previously published spectral data of authentic (+)-zizaene standard [28]. This proves that the khusimol-precursor is the main product of this enzymatic *in vivo* biotransformation. The additional smaller peaks 2 and 3 could not be identified, even in samples that were highly concentrated. Nevertheless, the presence of the molecular ion peak (M^+) *m/z* 204 strongly suggests that these compounds are sesquiterpenes.

No authentic standard of (+)-zizaene was available. Concentrations were determined as (−)-α-cedrene equivalents, using a calibration (Figure S9) of the commercial authentic standard (Sigma–Aldrich, USA). The structural strongly related α-cedrene was chosen for quantification purposes, due to its similar elementary composition ($C_{15}H_{24}$) in respect to zizaene. This results in an equivalent peak integral for the FID detector, as this detection method is proportional to the carbon content of the analyte.

3.3. Identification of enzyme kinetic parameters

Enzyme kinetics of the purified synthase were determined by calculating initial reaction rates after incubation with varying

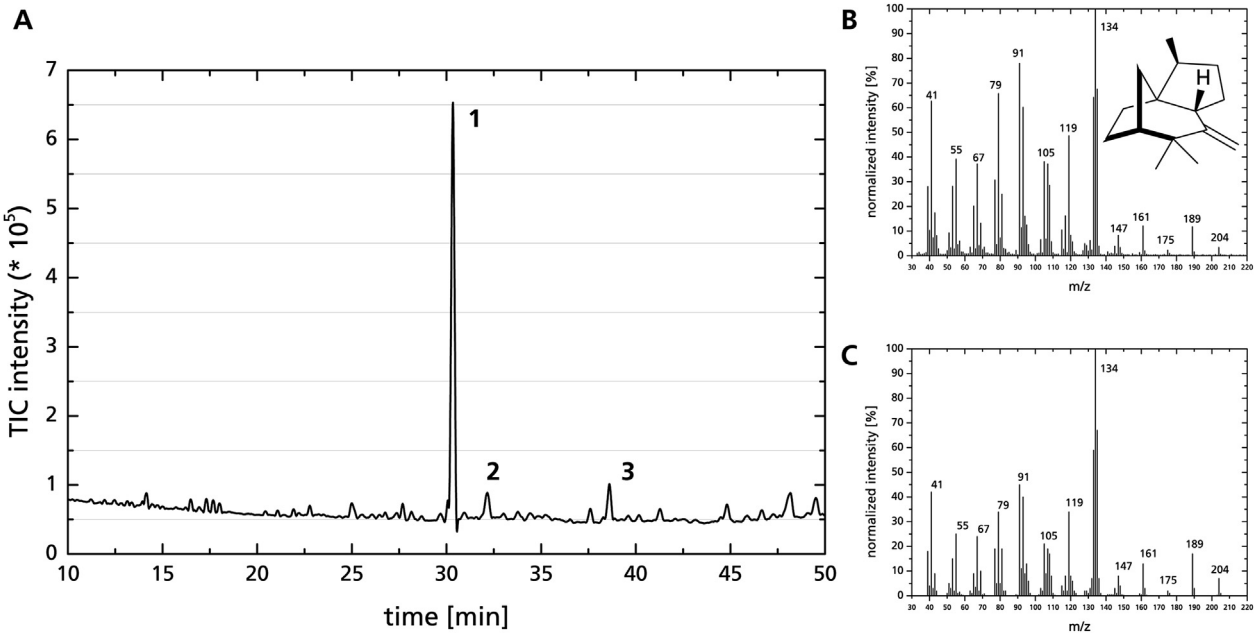

Fig. 3. (A) GC–MS chromatogram showing analyzed organic phase enriched with conversion products of recombinant zizaene synthase. (B) Mass spectrum peak 1 and (C) literature spectrum of authentic (+)-zizaene standard [28].

substrate concentrations. Product concentrations were measured directly by peak integration, based on the aforementioned α-cedrene calibration data using GC-FID. The optimal reaction temperature of 30 °C was identified in preliminary tests, using low FDP substrate concentrations. For rates to be in the linear range, a constant enzyme concentration of 0.11 μM was used in all set-ups. Products were trapped in an overlaid iso-octane phase. Hydrocarbons still present in the buffer phase were further extracted for 30 s by vigorous mixing, which additionally stopped enzyme catalysis due to precipitation of the protein by the organic solvent.

Reaction slopes were determined at least in triplicates for all substrate concentrations and the assumed Michaelis–Menten model fitted to the data (Fig. 4) by non-linear curve fitting using the Levenberg–Marquardt algorithm (least squares). The calculated K_M = 1.111 μM (±0.113) is relatively low, signifying a high binding affinity of the synthase to its substrate. Small K_M values are

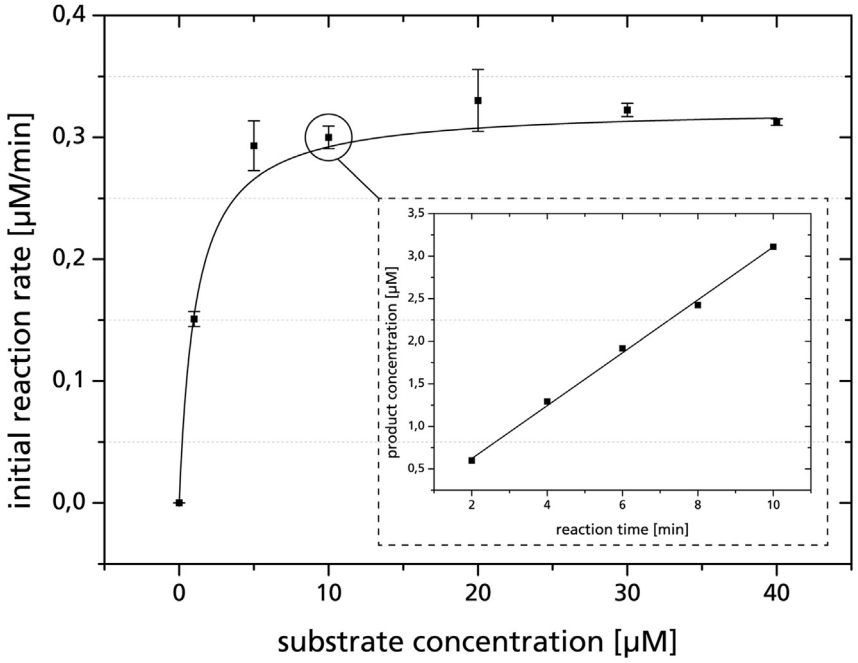

Fig. 4. Michaelis–Menten fitted plot, initial rate of reaction against increasing substrate concentrations. Each data point represents at least three individual linear regression plots with product concentrations determined at five time points (inserted graph).

Table 1
Compilation of kinetic data obtained in this study, comparison to some related STPSs. ND = not determined in source.

	Organism	T_{opt} [°C]	K_m [μM]	K_m SD (±)	V_{max} [μM min^{-1}]	V_{max} SD (±)	K_{cat} [min^{-1}]	K_{cat} [s^{-1}]	K_{cat}/K_m [M^{-1} s^{-1}]	Enzyme conc. [μM]	Specific activity [U mg^{1}]
(+)-zizaene synthase (this study)	*C. zizanoides*	30	1.111	0.113	0.3245	0.0035	2.95	0.049	4.43×10^4	0.11	0.0454
5-epi-aristolochene synthase [30]	*N. tabacum*	30	2.3	0.4	ND	ND	2.9	0.048	2.1×10^4	ND	ND
(S)-beta-bisabolene synthase [31]	*Z. mays*	30	2.1	ND	ND	ND	ND	ND	ND	ND	ND
vetispiradiene synthase [32]	*H. muticus*	30	0.7	0.4	ND	ND	2.4	0.04	ND	0.05	ND

typical for rather high molecular weight substrates like FDP, especially when large attachments like phosphate are present in the molecule [33]. The maximum reaction rate v_{max} = 0.3245 μM min^{-1}(±0.0035) and the derived turnover number k_{cat} = 2.95 min^{-1} are rather slow in comparison to many other described enzyme. Considering the complex reaction catalyzed, as well as the fact that the (+)-zizaene synthase belongs to the plant secondary metabolism, the turnover number lies in the typical range and is comparable to other related STPSs (Table 1). Enzymes of the secondary metabolism are usually several orders of magnitude slower than enzymes of the central metabolism, probably because the selection pressure for efficient catalysis is very low as the products or the amount of product are often not essential for the organism [33].

This study signifies that soluble expression of a STPS in *Escherichia coli* can be successfully achieved by fusion to a SUMO moiety as well as induction at low temperatures. These strategies might be transferable to novel hard-to-express terpene synthases discovered in the future. Purification of synthase was achieved to apparent homogeneity, employing a one-step IMAC affinity chromatography. The sesquiterpene (+)-zizaene is the sole product of the synthase, identified by GC–MS. Evaluated enzyme parameters like pI, substrate binding affinity, as well as turnover numbers are comparable to previously described cyclases of the STPS subclass. To enable efficient biocatalyzed production of the khusimol precursor (+)-zizaene, it might be feasible to optimize the catalytic efficiency by mutations strategies like directed evolution.

Conflict of interest

None.

Acknowledgments

The authors would like to thank Ralf Günter Berger and Ulrich Krings from the Institute of Food Chemistry in Hannover for kind support with GC–MS analytics. We also thank Ursula Rinas and Manfred Nimtz from the Helmholtz Centre for Infection Research in Braunschweig for providing the MALDI-PMF analysis. This study was funded by the European Union as part of the EFRE (European Regional Development Fund) project “Refinement of plant resources” (ZW 8-80130940).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.053.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.02.053.

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