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

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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 *E. 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 = $4.43x10^4$ $M^{-1}s^{-1}$ 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.

Keywords

Terpene synthase, Sesquiterpenes, Zizaene, Khusimene, Enzyme kinetics, SUMO[1]

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

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 1,500 – 1,700 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.

Figure 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 modelled from aa sequence using I-Tasser algorithm [5] and rendered in QuteMol [6].

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 (Figure 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.

Materials & Methods

Cloning of expression constructs

The unannotated cDNA sequence GenBank: HI931360 [17] was carefully codon-optimized for expression in *E. coli* K12 derivative strains using a guided-random approach [18]. The optimized sequence (1,668 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 5x 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).

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 10x 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 x 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 x g, 4 °C, 60 min), the soluble supernatant was directly used in SDS-PAGE analysis. Insoluble 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.

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.

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].

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 88210 $M^{-1}cm^{-1}$) 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 – 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).

Results and discussion

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 (Figure 2, A+B). Inducer concentration had no significant impact on protein yield.

Figure 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.

It is suspected, that the rapid folding of a highly soluble and structurally compact SUMO moiety in *E. 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 (Figure 2, C).

With soluble enzyme yields of up to ~325 $mg \cdot L^{-1}$ vs. ~221 $mg \cdot L^{-1}$ (densitometric analysis, shake flask scale, TB medium), the SUMO-fusion strategy yielded superior amounts compared to cold-shock 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 (Figure 2, D+E). Purity of enzyme was >95 %, determined by densitometry. Western-Blots with His-epitope antibody (Figure 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).

Activity assays and identification of enzyme products

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

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 (Figure 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.

Identification of enzyme kinetic parameters

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

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

Figure 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).

Reaction slopes were determined at least in triplicates for all substrate concentrations and the assumed Michaelis-Menten model fitted to the data (Figure 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 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 *E. 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.

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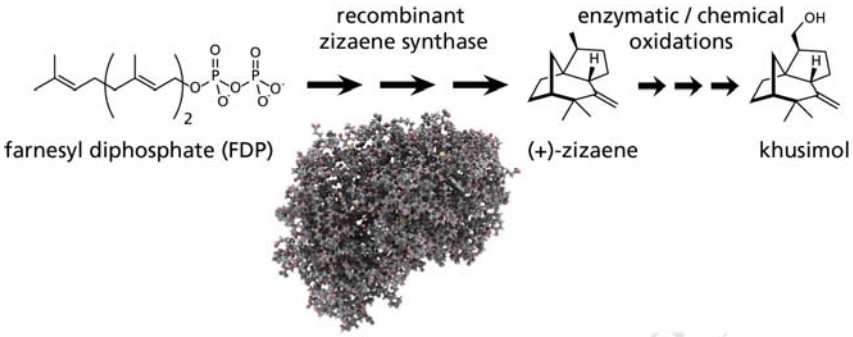
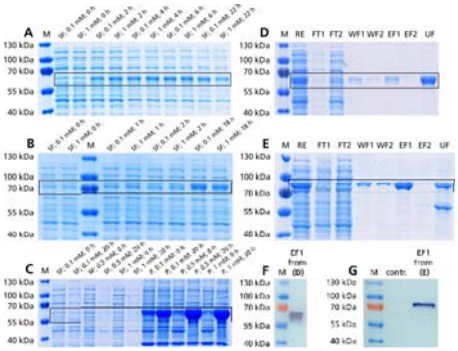
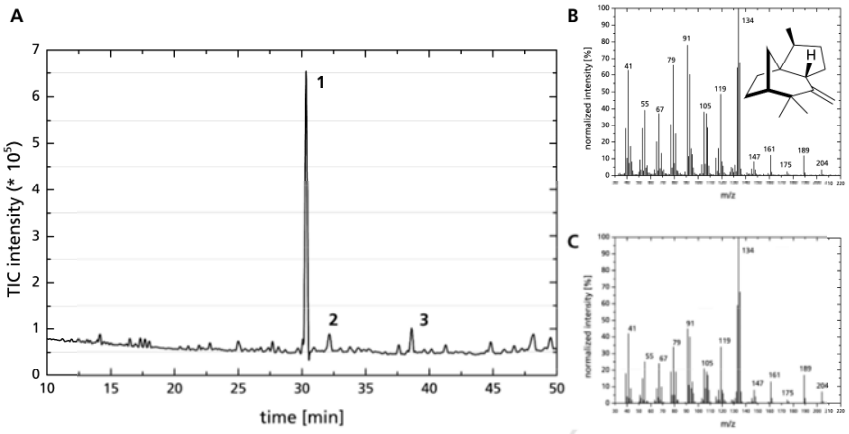
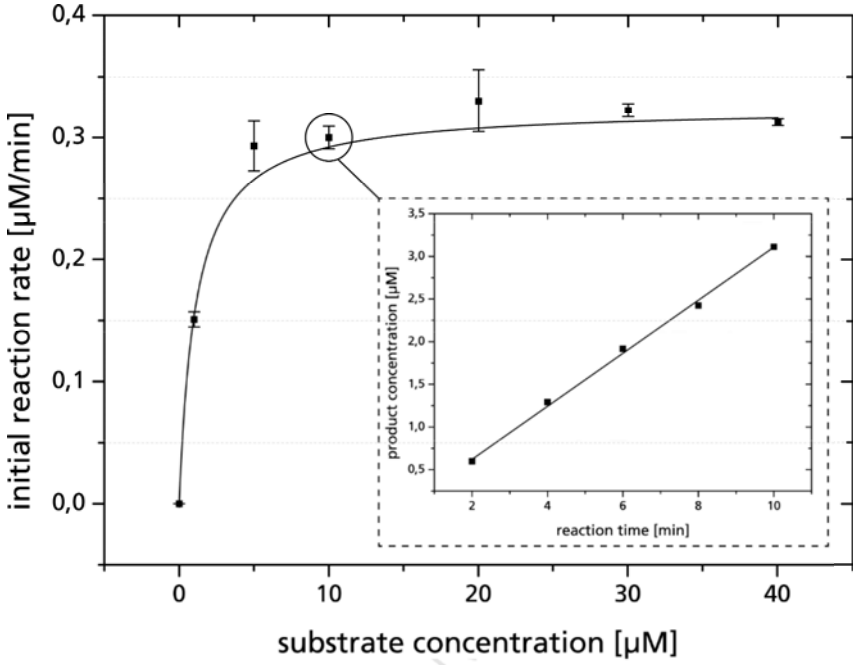
	organism	T_{opt} [°C]	K_m [µM]	K_m SD (±)	V_{max} [µM min^{-1}]	V_{max} SD (±)	K_{cat} [min^{-1}]	K_{cat} [s^{-1}]	K_{cat}/K_m [M^{-1} s^{-1}]	enzyme conc. [µM]	specific activity [U mg^{-1}]
(+)-zizaene synthase (this study)	*C. zizanoides*	30	1.111	0.113	0.3245	0.0035	2.95	0.049	$4.43x10^4$	0.11	0.0454
5-epi-aristolochene synthase [30]	*N. tabacum*	30	2.3	0.4	ND	ND	2.9	0.048	$2.1x10^4$	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



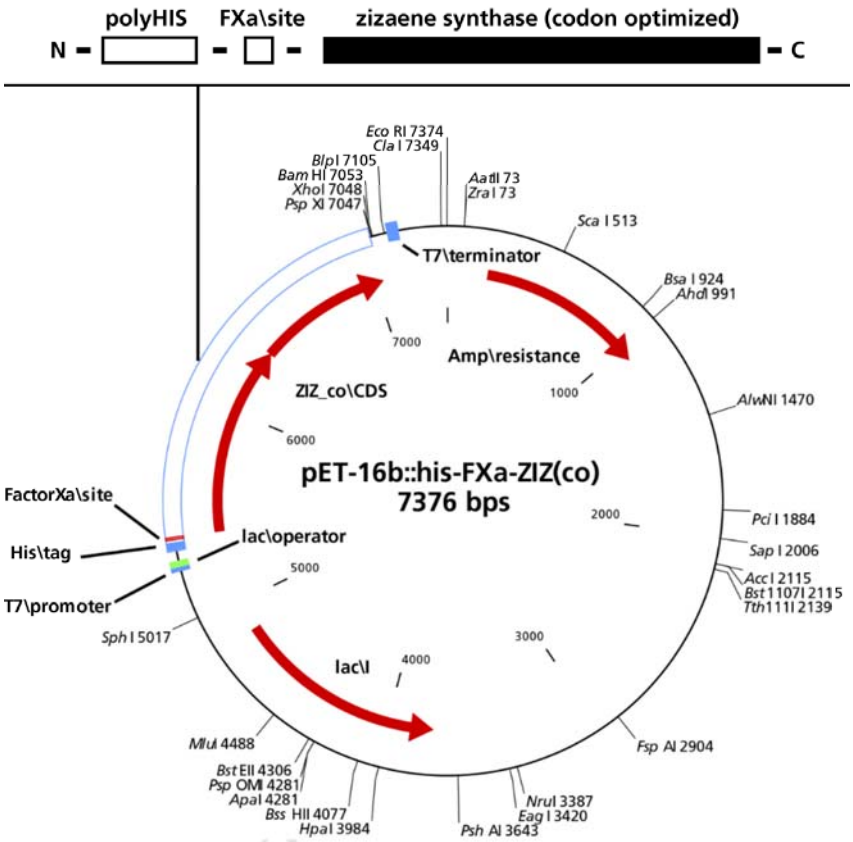


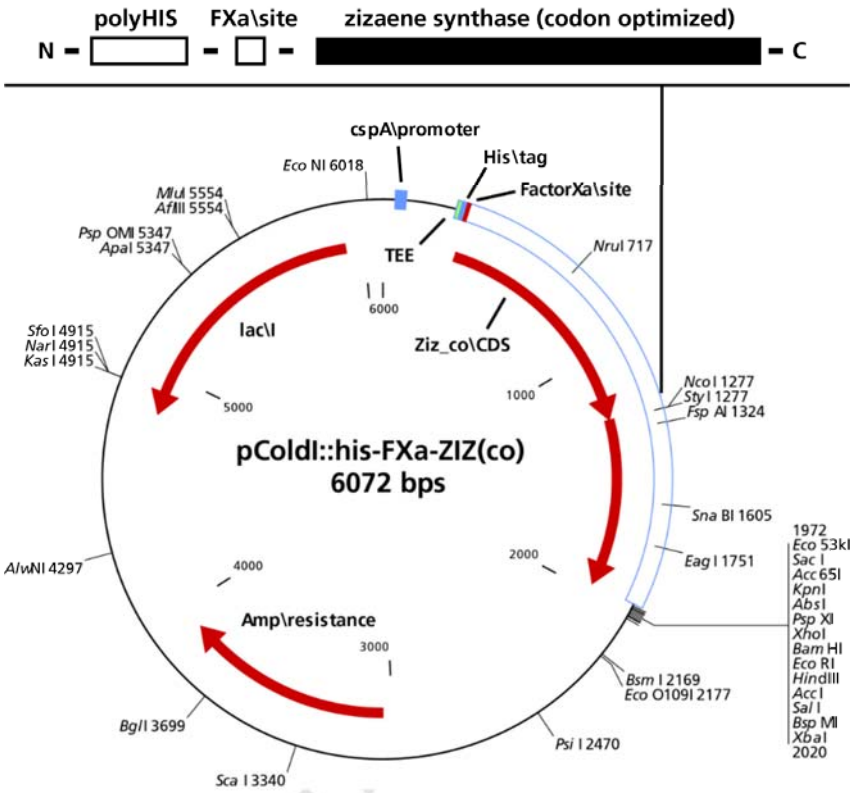


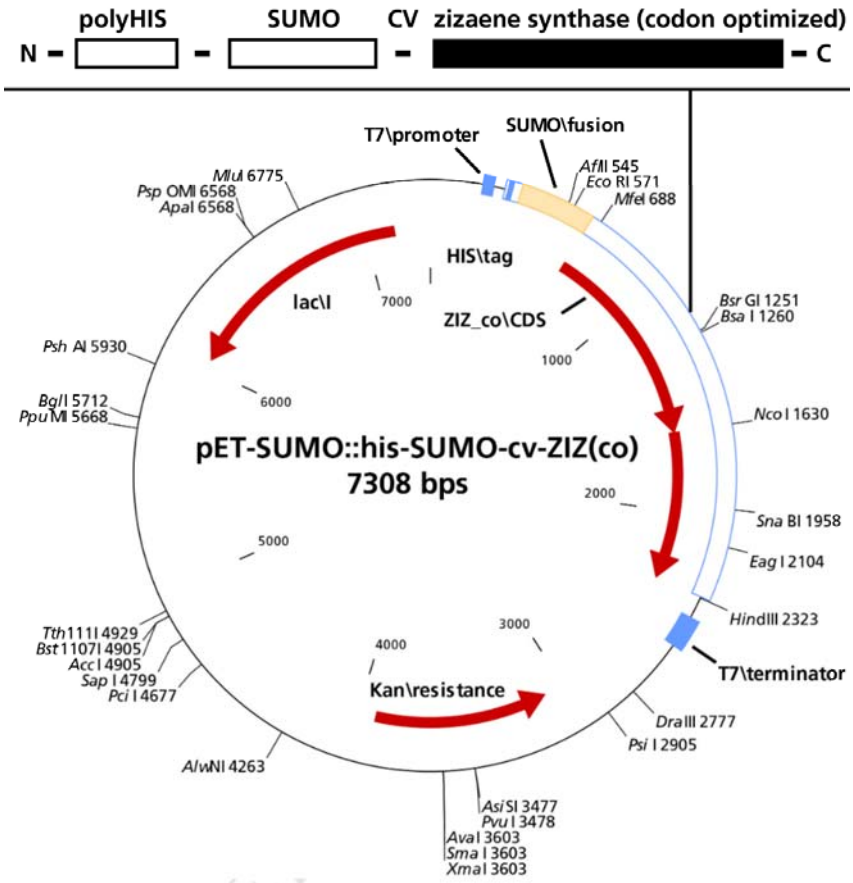

initial reaction rate [µM/min]
substrate concentration [µM]
product concentration [µM]
reaction time [min]

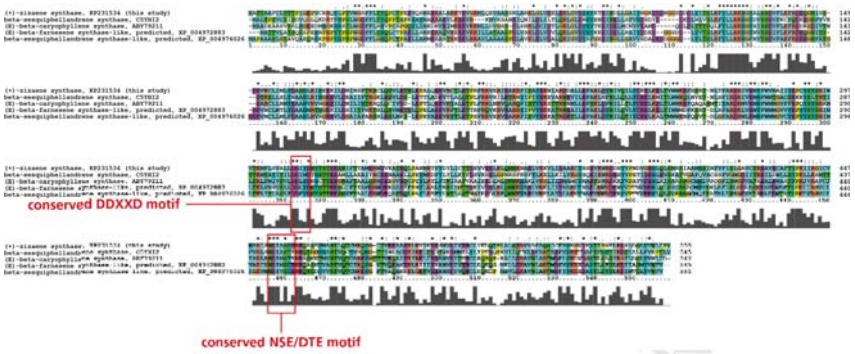
Highlights

- uncharacterized (+)-zizaene synthase from *C. zizanoides* was cloned and expressed
- fusion to SUMO and cold-shock induction enhanced soluble yields in *E. coli*
- Ni^{2+}-IMAC purification of the SUMO-fused and unfused enzyme
- (+)-zizaene identified as main cyclization product by GC-MS
- enzyme kinetic parameters comparable to related sesquiterpene synthases


polyHIS
FXa\site
zizaene synthase (codon optimized)
N
C
pET-16b::his-FXa-ZIZ(co)
7376 bps
T7\terminator
Amp\resistance
ZIZ_co\CDS
lac\operator
lac\I
FactorXa\site
His\tag
T7\promoter
Eco RI 7374
Cla I 7349
BlpI 7105
BamHI 7053
XhoI 7048
Psp XI 7047
AatII 73
ZraI 73
Sca I 513
Bsa I 924
AhdI 991
AlwNI 1470
Pci I 1884
Sap I 2006
AccI 2115
Bst1107I 2115
Tth111I 2139
Fsp AI 2904
NruI 3387
EagI 3420
Psh AI 3643
HpaI 3984
Bss HII 4077
ApaI 4281
Psp OMI 4281
BstEII 4306
MluI 4488
Sph I 5017
7000
1000
6000
2000
5000
3000
4000


polyHIS
FXa\site
zizaene synthase (codon optimized)
N
C
cspA\promoter
His\tag
FactorXa\site
Eco NI 6018
Mlu I 5554
AflIII 5554
Psp OMI 5347
ApaI 5347
TEE
NruI 717
6000
lac\I
Ziz_co\CDS
Sfo I 4915
NarI 4915
Kas I 4915
5000
1000
NcoI 1277
Sty I 1277
Fsp AI 1324
pColdI::his-FXa-ZIZ(co)
6072 bps
Sna BI 1605
1972
Eco 53kI
Sac I
Acc 65I
KpnI
Abs I
Psp XI
XhoI
Bam HI
Eco RI
HindIII
Acc I
Sal I
Bsp MI
XbaI
2020
AlwNI 4297
4000
2000
Eag I 1751
Amp\resistance
3000
Bsm I 2169
Eco O109I 2177
BglI 3699
Psi I 2470
Sca I 3340


polyHIS
SUMO
CV
zizaene synthase (codon optimized)
N
C
T7\promoter
SUMO\fusion
AflII 545
EcoRI 571
MfeI 688
MluI 6775
PspOMI 6568
ApaI 6568
HIS\tag
lacI
7000
ZIZ_co\CDS
1000
BsrGI 1251
BsaI 1260
PshAI 5930
6000
BglI 5712
PpuMI 5668
NcoI 1630
pET-SUMO::his-SUMO-cv-ZIZ(co)
7308 bps
2000
SnaBI 1958
5000
EagI 2104
HindIII 2323
3000
4000
T7\terminator
Tth111I 4929
Bst1107I 4905
AccI 4905
SapI 4799
PciI 4677
Kan\resistance
DraIII 2777
PsiI 2905
AlwNI 4263
AsiSI 3477
PvuI 3478
AvaI 3603
SmaI 3603
XmaI 3603

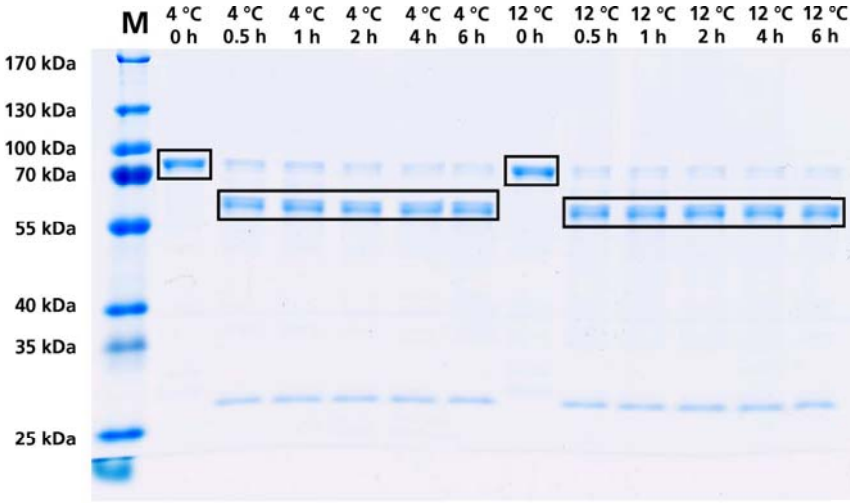


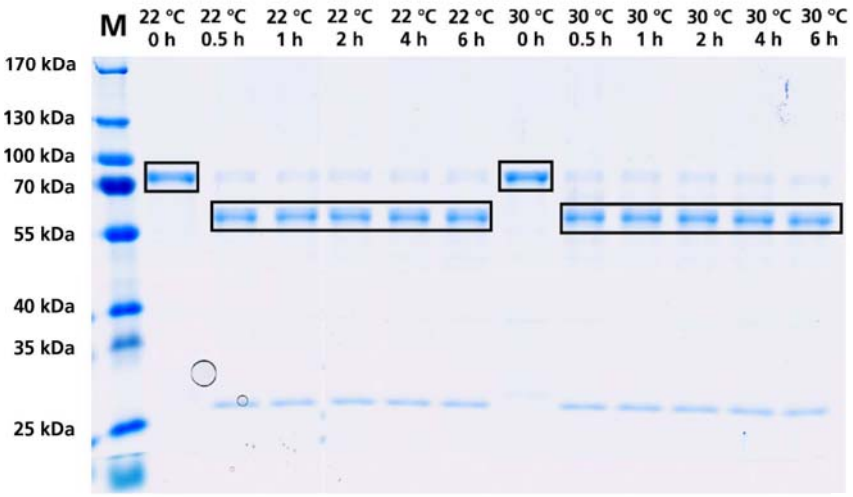
Translated sequence of pET-SUMO::ZIZ(co) →673 AA

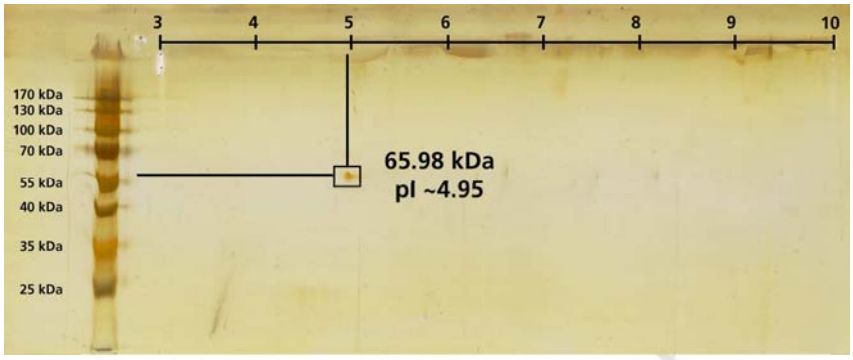
```
        10         20         30         40         50         60
MGSSHHHHHH GSGLVPRGSA SMSDSEVNQE AKPEVKPEVK PETHINLKVS DGSSEIFFKI
        70         80         90        100        110        120
KKTTPLRRLM EAFAKRQGKE MDSLRFLYDG IRIQADQTPE DLDMEDNDII EAHREQIGGA
       130        140        150        160        170        180
TTAAFCLTTT PIGEPVCRRQ YLPTVWGSFF LTYQPCTPEE VQSMEERALA KKTEVGRMLQ
       190        200        210        220        230        240
EVAASSNLAR KLGLVDELER LGVDYHYKTE INDLLGAIYN GKDDDNGGSD DDLYITSLKF
       250        260        270        280        290        300
YLLRKHGYAL SSDVFLKFRD EQGNISSDDV KCLIMLYDAS HLRIHEEKIL DNINSFTKSC
       310        320        330        340        350        360
LQSVLETNLE PALQEEVRCT LETPRFRRVE RIEAKRFISA YEKNIARDDA LLEFARLDYN
       370        380        390        400        410        420
IVQILYCKEL KELTVWWKEF HSRTNLTFAR DRIVEMYFWV MAIIYEPCYS YSRIWVTKMF
       430        440        450        460        470        480
LSVALLDDIY DNYTSTEESN IFTTAMEKWD VKATEQLPAN MRTFYDYLIC TTDEVVEELK
       490        500        510        520        530        540
LQNNKNAELV KKVLIDAAKC YHSEVKWRDD HYVPNDVGEH LQLSMRSIAA MHSINFVFIS
       550        560        570        580        590        600
LGAVCTREAV ECAFTYPKII RGICVHARIS NDIASEREQ ASEHMASTLQ TCMKQYGITV
       610        620        630        640        650        660
EEAAEKLRVI NEESWMDIVE ECLYKDQYPL ALSEVVAFA QSICFMYNGV DKYTIPSKLK
       670
DSLDSLYVNL IPV
```

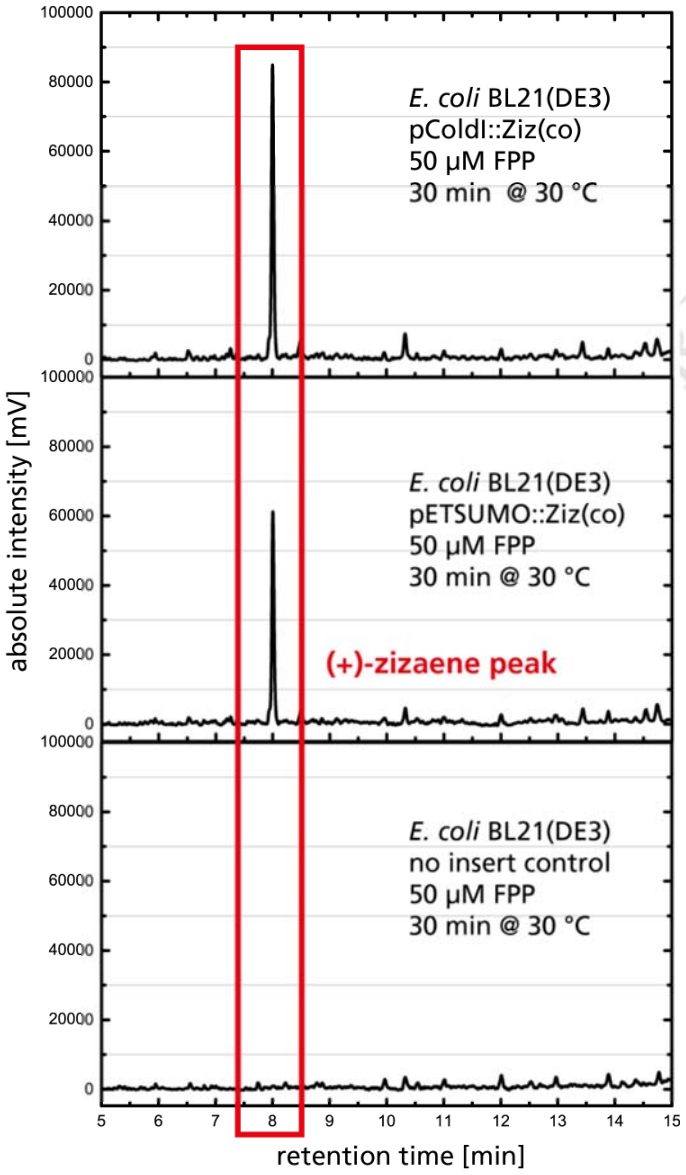
Protein digested with Trypsin. All cysteins have been treated with Iodoacetamide to form carbamidomethyl-cystein.

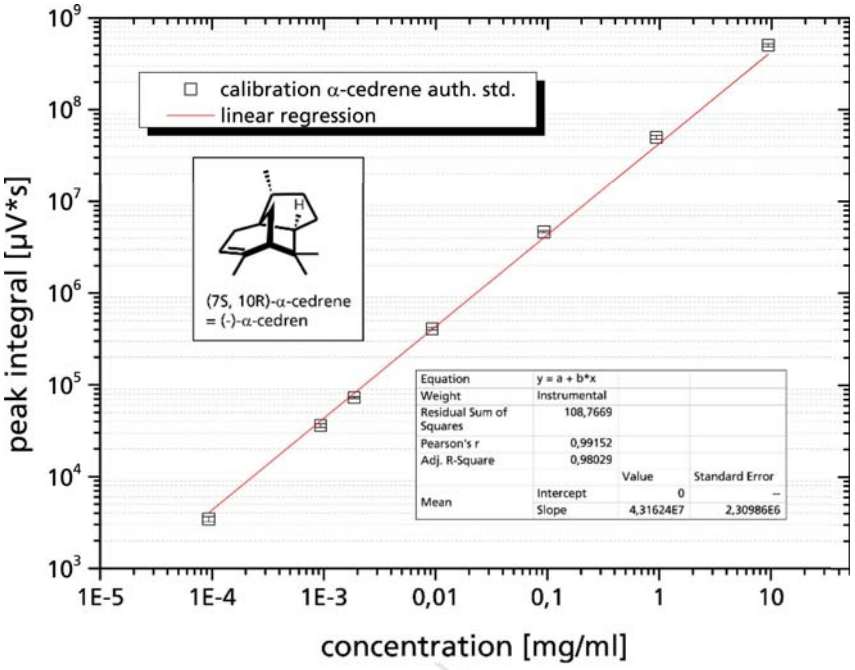
peptide mass (detected)	position	peptide sequence
2568.1518	AA 93-114	IQADQTPEDLDMEDNDIIEAHR
1491.7446	AA 272-283	CLIMLYDASHLR
1389.7154	AA 178-190	MLQEVAASSNLAR
1336.6895	AA 246-257	HGYALSSDVFLK
1314.6034	AA 548-558	EAVECAFTYPK
1215.5892	AA 49-59	VSDGSSEIFFK
1191.6004	AA 626-635	DQYPLALSER
1130.5622	AA 453-462	ATEQLPANMR
1049.5262	AA 348-356	DDALLEFAR
994.4992	AA 201-208	LGVDYHYK


M
4 °C 0 h
4 °C 0.5 h
4 °C 1 h
4 °C 2 h
4 °C 4 h
4 °C 6 h
12 °C 0 h
12 °C 0.5 h
12 °C 1 h
12 °C 2 h
12 °C 4 h
12 °C 6 h
170 kDa
130 kDa
100 kDa
70 kDa
55 kDa
40 kDa
35 kDa
25 kDa


M
22 °C 0 h
22 °C 0.5 h
22 °C 1 h
22 °C 2 h
22 °C 4 h
22 °C 6 h
30 °C 0 h
30 °C 0.5 h
30 °C 1 h
30 °C 2 h
30 °C 4 h
30 °C 6 h
170 kDa
130 kDa
100 kDa
70 kDa
55 kDa
40 kDa
35 kDa
25 kDa




E. coli BL21(DE3)
pColdI::Ziz(co)
50 µM FPP
30 min @ 30 °C
E. coli BL21(DE3)
pETSUMO::Ziz(co)
50 µM FPP
30 min @ 30 °C
(+)-zizaene peak
E. coli BL21(DE3)
no insert control
50 µM FPP
30 min @ 30 °C
absolute intensity [mV]
retention time [min]


calibration α-cedrene auth. std.
linear regression
(7S, 10R)-α-cedrene
= (-)-α-cedren
peak integral [µV*s]
concentration [mg/ml]
Equation y = a + b*x
Weight Instrumental
Residual Sum of Squares 108,7669
Pearson's r 0,99152
Adj. R-Square 0,98029
Value Standard Error
Mean Intercept 0 –
Slope 4,31624E7 2,30986E6

No.	Plasmid construct	Description	Primer sequence (5' – 3')
1	pET-16b::his-FXa-ZIZ(co)	pET16b_VEC_FWD	CATATGCTCGAGGATCCG
2	pET-16b::his-FXa-ZIZ(co)	pET16b_VEC_REV	ACGACCTTCGATATGGCC
3	pET-16b::his-FXa-ZIZ(co)	ZIZ(co)_String1_FWD	CCATATCGAAGGTCGTGCAACCACTGCTGCGTTTTGCTTA
4	pET-16b::his-FXa-ZIZ(co)	ZIZ(co)_String1_REV	GGCAGTCGTGAAGATATTAGACTCCTCGGTGC
5	pET-16b::his-FXa-ZIZ(co)	ZIZ(co)_String2_FWD	TATCTTCACGACTGCCATGGAGCGGTGGGACG
6	pET-16b::his-FXa-ZIZ(co)	ZIZ(co)_String2_REV	GATCCTCGAGCATATGTCACACCGGAATCAGATTTACATA
7	pColdI::his-FXa-ZIZ(co)	pColdI_VEC_FWD	CATATGGAGCTCGGTACCCT
8	pColdI::his-FXa-ZIZ(co)	pColdI_VEC_REV	CCTACCTTCGATATGATGATGATG
9	pColdI::his-FXa-ZIZ(co)	ZIZ(co)_String1_FWD	CATATCGAAGGTAGGGCAACCACTGCTGCG
10	pColdI::his-FXa-ZIZ(co)	ZIZ(co)_String1_REV	GCTCCATGGCAGTCGTGAAGATATTAGACTCCTCGGTG
11	pColdI::his-FXa-ZIZ(co)	ZIZ(co)_String2_FWD	AGTCTAATATCTTCACGACTGCCATGGAGCG
12	pColdI::his-FXa-ZIZ(co)	ZIZ(co)_String2_REV	ACCGAGCTCCATATGTCACACCGGAATCAGATTTACA
13	pETSUMO::his-SUMO-cv-ZIZ(co)	pETSUMO_VEC_FWD	AGACAAGCTTAGGTATTTATTCG
14	pETSUMO::his-SUMO-cv-ZIZ(co)	pETSUMO_VEC_REV	ACCACCAATCTGTTCTCTG
15	pETSUMO::his-SUMO-cv-ZIZ(co)	ZIZ(co)_String1_FWD	GAACAGATTGGTGGTGCAACCACTGCTGCG
16	pETSUMO::his-SUMO-cv-ZIZ(co)	ZIZ(co)_String1_REV	GCTCCATGGCAGTCGTGAAGATATTAGACTCCTCGGTG
17	pETSUMO::his-SUMO-cv-ZIZ(co)	ZIZ(co)_String2_FWD	AGTCTAATATCTTCACGACTGCCATGGAGCG
18	pETSUMO::his-SUMO-cv-ZIZ(co)	ZIZ(co)_String2_REV	TACCTAAGCTTGTCTTCACACCGGAATCAGATTTACA