Biotechnol Lett (2009) 31:565–569
DOI 10.1007/s10529-008-9903-2

ORIGINAL RESEARCH PAPER

Squalene-hopene cyclase (Spterp25) from *Streptomyces peucetius*: sequence analysis, expression and functional characterization

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Received: 23 October 2008 / Revised: 27 November 2008 / Accepted: 4 December 2008 / Published online: 31 December 2008

Abstract Squalene-hopene cyclase, which catalyzes the complex cyclization of squalene to the pentacyclic triterpene, hopene, is a key enzyme in the biosynthesis of hopanoids. The deduced amino acid sequence of the *Streptomyces peucetius* gene (*spterp25*) had significant similarity to other prokaryotic squalene-hopene cyclases. Like other triterpene cyclases, the *S. peucetius* squalene-hopene cyclase contains eight so-called QW-motifs with an aspartate-rich domain. The 2,025-bp squalene-hopene cyclase-encoding gene was expressed in *Escherichia coli* BL21(DE3)pLySs, and the in vitro activity of the recombinant cyclase was demonstrated using purified membrane protein. The cyclization product hopene was identified by gas chromatography/mass spectrometry (GC/MS).

Keywords Heteroexpression · Hopanoids · Squalene-hopene cyclase · *Streptomyces peucetius*

Introduction

Hopanoids are pentacyclic triterpenoid lipids found in a wide range of Gram-positive and Gram-negative bacteria (Sahm et al. 1993).They are synthesized from isopentenyl units that are formed through two different metabolic routes leading to isopentenyl diphosphate (Meyer et al. 2003). To date, the most extensively studied triterpene cyclase is squalene-hopene cyclase (SHC) from the thermophilic bacterium *Alicyclobacillus acidocaldarius* (Wendt et al. 1997; Reinert et al. 2004). Squalene is an immediate precursor in hopanoid biosynthesis that is formed by six isoprene (C5) units. The key enzyme in the hopanoid biosynthesis is SHC, which catalyzes the cyclization of the linear triterpenoid squalene to hopene (Fig. 1).

Hopanoids have a structure similar to that of sterols (Qurisson et al. 1979).They are cyclized from squalene (Anding et al. 1976), whereas sterols are synthesized from squalene epoxide (Corey et al. 1966; van Tamelen et al. 1966). Hopanoids are involved in regulating membrane fluidity and stability; they are comparable to sterols in the membranes of eukaryotes, but the structural variants of hopanoids indicate that they may have some other interesting functions (Kannenberg and Poralla 1999).

The genome of *S. peucetius* was recently determined (Parajuli et al. 2004). Through genome sequencing of *S. peucetius*, we found one open reading frame of 2,025 bp, named *spterp25*, which encodes a putative protein of 674 aa with significant similarity to *S. coelicolor* A3(2) (SCO6764), *S. avermitilis* MA-4680 (SAV1650), and *Alicyclobacillus acidocaldarius* (SHC). We expect that Spterp25 would act as a

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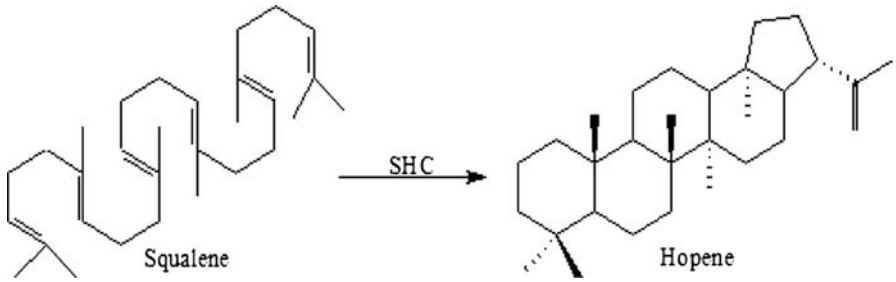

Fig. 1 Reaction scheme of the cyclization of squalene to hopene by squalene-hopene cyclase

squalene-hopene cyclase if heterologously expressed because sequence alignment of the deduced amino acids (aa) of the Spterp25 protein with the known squalene-hopene cyclases provides strong bootstrap support for this activity. We cloned and heterologously expressed Spterp25 in *Escherichia coli* BL21(DE3) pLySs and performed in vitro enzyme assays to functionally characterize the *shc* as the first step in an examination of hopanoid biosynthesis.

Materials and methods

Bacterial strains, plasmids and culture conditions

Streptomyces peucetius was grown in 50 ml R2YE medium (5% w/v sucrose, 0.02% potassium sulfate, 1% $MgCl_2$, 1% glucose, 0.5% yeast extract, and 0.01% Difco Casamino acids) at 28°C in a shaking incubator for 4–5 days in order to isolate genomic DNA. *E. coli* XL1-Blue MRF and *E. coli* BL21 (DE3)pLySs (Stratagene, La Jolla, CA) were used as hosts for plasmid preparation and expression, respectively. pET32a(+) (Novagen, USA) was used as an expression vector. *E. coli* strains were grown in Luria Bertani (LB) medium, both in liquid and agar plates, with appropriate antibiotics for selection (ampicillin and chloramphenicol at 100 μg/ml and 50 μg/ml, respectively). Oligonucleotides were synthesized by GenoTech (Daejeon, Korea). Restriction enzymes were purchased from Takara company (Japan). All chemicals were purchased from Sigma unless otherwise stated. Database searches were performed by using the BLAST, FASTA and CLUSTALW programs.

DNA manipulation

Genomic DNA was isolated from *S. peucetius* using the Kirby mix procedure (Kieser et al. 2000). Two primers SHC-F (5′-ACC **GAA TTC** TGC AAC GAA GGG GAG AAC ATG-3′) and SHC-R (5′-AAT **AAG CTT** ACG GCT CCC GGG AGT CCC TCA-3′), were used to amplify *shc* (restriction sites are indicated by the underlined letters).The amplification was performed in a total volume of 20 μl and the PCR product (2,025 bp) was cloned into the *Eco*RI and *Hin*dIII sites of pET32a(+) to generate pSPSHC (Fig. 2a). To verify that no mutations had occurred during the PCR amplification, all of the PCR products were cloned into a pGEM-T Easy vector and sequenced prior to cloning into the expression vector. PCR was performed using a thermocycler (Takara, Japan) under the following conditions: denaturation at 94°C for 7 min, 30 cycles of annealing at 62°C for 1 min, and polymerization at 72°C for 1 min.The recombinant vector was introduced into *E. coli* BL21(DE3) pLySs by heat- pulse transformation.

Enzyme preparation

E. coli BL21(DE3) pLySs harboring pSPSHC was grown in 3 ml LB medium supplemented with ampicillin (100 μg/ml) and chloramphenicol (50 μg/ml) at 37° for 8 h, transferred into 50 ml LB medium supplemented with the appropriate antibiotics and grown at 37°C to an OD_{600} of 0.6. IPTG was then added to give 1 mM and the culture was re-incubated at 20°C for 20 h. Cells were harvested at 6,000*g* for 12 min, washed twice with cold buffer (100 mM Tris/HCl, 250 mM saccharose, 20 mM ascorbic acid, pH 8.0), and then re-suspended with 1 ml of the same buffer containing 1% (v/v) Triton X-100. All subsequent procedures were carried out at 4°C. After cell breakage, the supernatant and pellet fractions were separated by centrifugation at 12,000*g* for 20 min. The soluble protein was purified by Co^{2+}-affinity chromatography (Talon Purification Kit, Clontech, Takara Bio Company, USA) according to

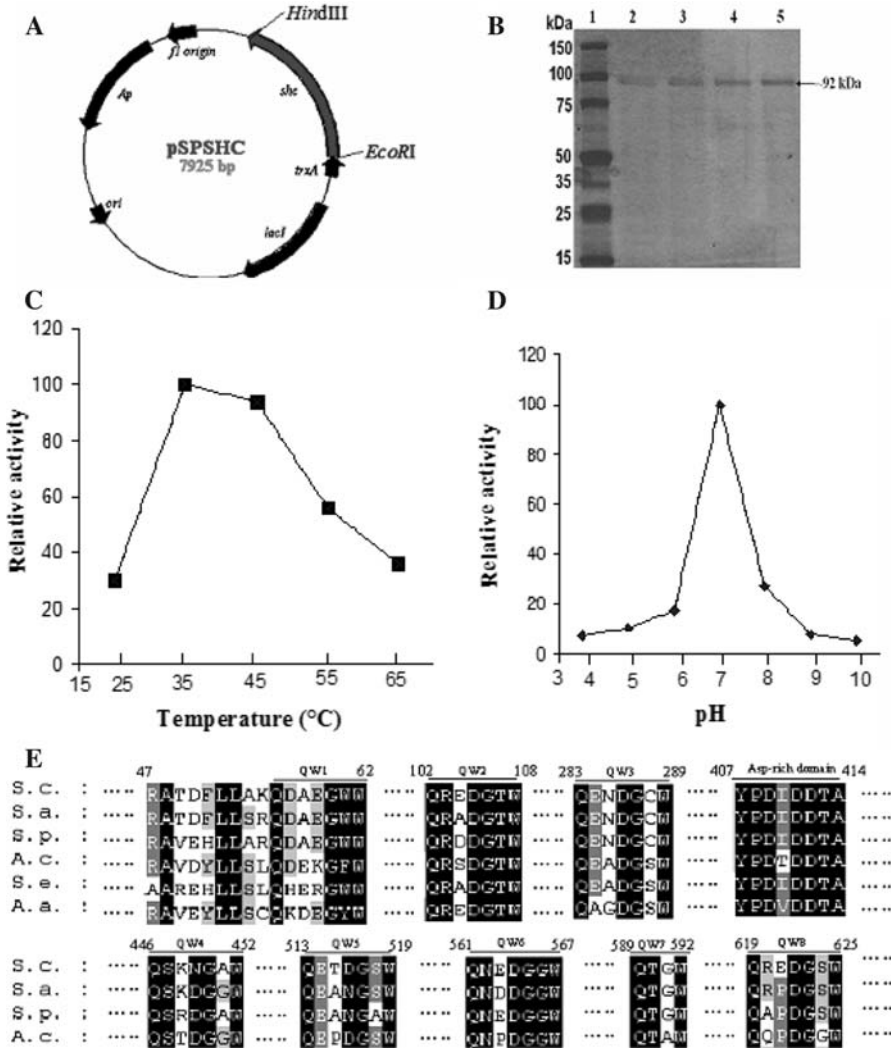

Fig. 2 **a** Construction of expression recombinant pSPSHC, *shc* from *S. peucetius* cloned into the pET32a(+) vector. **b** SDS-PAGE analysis of recombinant overexpressed Spterp25 visualized by Coomassie staining. Lane 1, molecular weight markers; lane 2–5, purified elution fractions containing Spterp25. **c** Effect of temperature on the relative activity of the recombinant *S. peucetius* SHC. SHC was assayed at temperature range from 25 to 65°C, pH 6.8 with squalene as a substrate. **d** Effect of pH on the relative activity of the recombinant *S. peucetius* SHC. SHC was assayed over the pH range from 3.0 to 10.0, at 35°C with squalene as a substrate. **e** Sequence alignment of the deduced *S. peucetius* (S.p.) SHC protein with the known squalene-hopene cyclases. The sequence was compared with S.c., *S. coelicolor*A3 (2) (GenBank accession no. NP_630836); S.a., *S. avermitilis* MA-4680 (GenBank accession no. NP_822826); A.c., *Acidothermus cellulolyticus* 11 B (GenBank accession no. YP_873455); S.e., *Saccharopolyspora erythraea* NRRL 2338 (GenBank accession no. YP_001106521); and A.a., *Alicyclobacillus acidocaldarius* (GenBank accession no. BAA25185). Eight repetitive QW-motifs and the conserved aspartate-rich domain are shown. Numbers are given from the *S. peucetius* amino acid sequence

the manufacturer's instructions. The purified fractions were desalted by dialysis and concentrated by Centricon (Ultracel YM-10, Millipore). The purity of recombinant protein was evaluated by 12% (v/v) DS-PAGE.

Enzyme assay and in vitro analysis

The reaction mixture contained 20 µl 1 M sodium citrate (pH 6.8), 20 µl substrate (100 µM squalene, 1% (v/v) Triton X-100, 100 mM sodium citrate

pH 6.8), 30 µl purified enzyme, and 30 µl distilled water in a total volume of 100 µl and was held for 5 h at 35°C *E. coli* harboring only the vector was employed as a negative control. The reaction mixture was extracted with 200 µl hexane/2-propanol (3:2, v/v). The combined organic layer was collected and evaporated, and the recovered lipids were re-dissolved in the same solvent. The concentrated extract, 1–5 µl, was subjected to GC/MS analysis (Agilent 5973 inert MSD, oven temperature was set from 250 to 320°C with temperature gradient of 4°C per minute increase followed by 20 min at 320°C).

Effect of temperature and pH on enzyme activity

The effect of temperature on the enzyme activity was determined from 25 to 65°C in the presence of squalene solubilized with 1% (v/v) Triton X-100 in sodium citrate buffer. The pH dependence of SHC activity was also determined at 35°C in sodium citrate buffer from pH 3 to 10.

Nucleotide sequence accession number

The sequence of *spterp25* reported here was deposited in the GenBank under accession number ACA52082.

Results and discussion

Comparison of the deduced amino acid sequence of the cloned ORF (*spterp25*) from *S. peucetius* revealed highly conserved residues that are typical of SHCs. It shares two striking amino acid motifs with other SHCs. First, a repetitive QW-motif with the consensus sequence (K/R)(G/A) X_{2-3} (F/Y/W) L X_3 QX_{2-5} G X W occurs eight times in *S. peucetius* SHC (Fig. 2e), which helps the enzyme to maintain its integrity (Chen 1997; Brody et al. 1997). Second, an aspartate-rich amino acid motif (Fig. 2e) with the consensus Y P D X D D T A is also highly conserved among SHCs. Similar aspartate-rich motifs also occur in enzymes that synthesize geosmin from farnesyl diphosphate, and

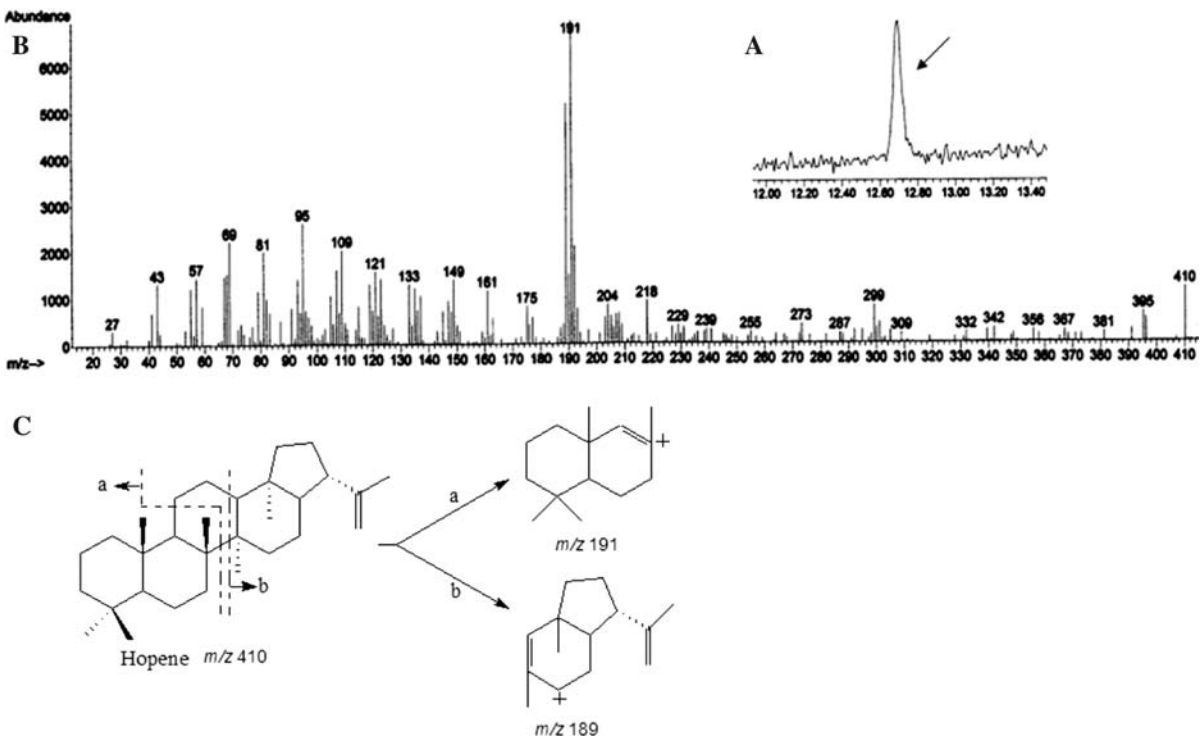

Fig. 3 GC–MS analysis of the assay product (hopene) produced from squalene by SHC. **a** GLC traces containing hopene (shown by arrow at r.t. 12.69 min.). **b** GLC–MS profile of hopene. (**c**) GLC–MS fractionation analysis of hopene. The mass peak at $m/z = 410$, base peak $m/z = 191$ and the fragmentation patterns are indicative for hopene

these motifs are involved in binding and/or stabilization of the diphosphate moieties via divalent cations (Mg^{2+} or Mn^{2+}) (Jiaoyang et al. 2007). In contrast, SHC activity does not depend on the presence of divalent cations and the substrate does not possess a pyrophosphate group. These negatively charged amino acids and conserved aromatic amino acid residues could stabilize intermediate carbocations during the cyclization process (Abe et al. 1993). Site-directed mutagenesis of the second and third aspartate residues of the YPDXDDTA motif of SHC from *Alicyclobacillus acidocaldarius* led to complete inactivation of the SHC, indicating the role of these residues in enzyme activity (Fiel et al. 1996; Sato and Hoshino 1999). Therefore, based on amino acid alignment and conserved domains, Spterp25 should function as a squalene-hopene cyclase.

The 2,025-bp *shc* was amplified and cloned into pET32a(+) expression vector at the *Eco*RI/*Hin*dIII sites to produce pSPSHC. Recombinant pSPSHC was transferred into *E. coli* BL21(DE3) pLySs and expression was optimized. The expression was induced by treatment with 1 mM IPTG for 20 h at 20°C. The soluble fraction was purified, desalted and concentrated as described in Materials and methods. The molecular weight of the recombinant Spterp25 was approx. 92 kDa according to SDS-PAGE analysis; this was consistent with the predicted molecular weight of Spterp25 plus his-tag (Fig. 2b). The activity of the enzyme was found maximum from 35 to 45°C (Fig. 2c) at pH 6.8 (Fig. 2d).

To demonstrate the predicted function of Spterp25, the enzyme reaction was carried out. The recombinant protein was incubated with squalene as described in the Materials and methods section, and the resulting product was analyzed by GC/MS. The pentacyclic product hopene (r.t. 12.69 min, $[M^+]$ m/z = 410, base peak m/z = 191) was identified by GC/MS (Fig. 3). *E. coli* extract harboring only vector was used as a control for the enzyme assay; no hopene was detected under these conditions, confirming that the cloned gene encodes the SHC.

The combined evidence from sequence analysis and enzymatic conversion conclusively established that *spterp25* gene encodes a squalene-hopene cyclase. This enzyme catalyzes the complex cyclization of squalene to the pentacycarbocyclic hopene and represents the key reaction in the biosynthesis of hopanoids.

Acknowledgments This study was supported by a grant of the National R&D Program for Cancer Control, Ministry of Health & Welfare, Republic of Korea (0720260).

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