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Analysis of Squalene Synthase Expression During the Development of *Ganoderma lucidum*

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Abstract The medicinal properties of *Ganoderma lucidum* have been recognized in China for many centuries. Active pharmaceutical components include triterpenes. To elucidate the molecular regulation of triterpene biosynthesis in this mushroom, a 57-base pair DNA fragment encoding the fourth conserved domain SQ-4 (SMGLFLQKTNIIRDYNEDL) of squalene synthase was synthesized and cloned into the expression vector pET-32a(+). The recombinant fusion protein induced by IPTG (isopropyl-β-D-thiogalactopyranoside) was overexpressed in the *Escherichia coli*. Using the purified recombinant fusion protein of 20.9 kDa, a specific polyclonal antibody was obtained from immunized rabbit. Expression of squalene synthase at different development stages of *Ganoderma lucidum* was analyzed.

Key words: Expression characteristics, *Ganoderma lucidum*, squalene synthase, triterpene

The fungus *Ganoderma lucidum* (Curtis: Fries) Karsten (Polyporaceae) has been widely used as a home remedy for minor disorders and to promote vitality and longevity [4, 15]. Current research focused on the isolation and understanding of the mechanism of its pharmaceutically active components [3, 4, 15]. Over 200 triterpenes have been isolated [1, 3, 24], which display cytotoxic [14], histamine release-inhibiting [12], angiotensin-converting enzyme-inhibiting [18], and cholesterol synthetic-inhibiting effects [13, 21]. Recently, it has also been shown that some triterpenes are active against the human immunodeficiency virus (anti-HIV) [16, 17]. However, little is known about the molecular mechanism of triterpene biosynthesis in *G. lucidum*. As secondary metabolites, triterpenes are synthesized in the mevalonate pathway [11, 25], where farnesyl diphosphate is converted to squalene, then to 2,3-oxidosqualene, and finally undergoes a series of cyclization, oxidation, and reduction reactions [11]. Squalene synthase (farnesyl-diphosphate:farnesyl diphosphate farnesyltransferase, EC 2.5.1.21) is the key enzyme in triterpene biosynthesis in which two molecules of farnesyl diphosphate (FPP) condense to form squalene. In eukaryotes, FPP is found at a multiple branch point in the isoprenoid biosynthetic pathway. Catalyzed by other enzymes, FPP can be transformed into ubiquinones, heme a, geranylated proteins, and dolichols [25]. Squalene synthase is involved in the step at a branch point in the mevalonate pathway, and there is a positive correlation between the expression level of squalene synthase and the amount of triterpenes produced. As a key enzyme in the triterpene biosynthetic pathway, squalene synthase has been cloned from yeast [9, 25], humans [10], rat [23], tobacco [5], *Arabidopsis thaliana* [22], and other species. Since squalene synthase is a membrane-bound enzyme [5], it is difficult to purify.

To elucidate the regulation of squalene synthase in the synthesis of triterpenes in *G. lucidum*, a DNA fragment encoding the fourth conserved region (SQ-4) of squalene synthase was synthesized and expressed as a fusion protein in *Escherichia coli*. An antibody to the conserved region was used to study the expression characteristics of squalene synthase in different stages of development in *G. lucidum*.

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MATERIALS AND METHODS

The strain of *G. lucidum* used in this study was obtained from the culture collection of the Edible Fungi Institute, Shanghai Academy of Agricultural Science. *E. coli* BL21(DE3), plasmid pET-32a(+) from Novagen Corporation, and DNA restriction enzymes were from Takara Corporation. Oligonucleotides were synthesized by Shanghai Genecore Corporation.

Synthesis of the DNA Fragment Encoding the Fourth Conserved Region of Squalene Synthase

Based on the comparative analysis of published amino acid sequences of squalene synthase in yeast [9, 25], humans [10], tobacco [5], and *Arabidopsis thaliana* [22], a 57-base pair DNA fragment (*SQ4*) encoding the fourth conserved region (SQ-4: SMGLFLQKTNIIRDYNEDL) of squalene synthase was designed according to *E. coli* preferred codon usage. Two primers were synthesized: primer 1 - 5'AAAA*CCATGG*GC AGC ATG GGC CTG TTT CTG CAG AAA ACC AAC ATC ATC CGT3' (containing a *Nco*I restriction site at the 5' end), and primer 2 - 5'AAAA*GAATTC* CAG ATC TTC GTT ATA ATC ACG GAT GAT GTT GGT TTT CTG3' (containing an *Eco*RI restriction site at the 5' end). The target DNA fragment was synthesized in 0.5-ml tubes containing 12 pmol each of primer 1 and primer 2; 1×Pfu DNA polymerase reaction buffer (Stratagene, U.S.A.), 0.2 mmol/l each of dATP, dCTP, dGTP, and dTTP; and 5 units of Pfu DNA polymerase in the final volume of 20 µl (Stratagene, U.S.A.). Reactions were run for 1 cycle of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 10 min in a Programmable Thermal Cycler PTC100 (MJ Research Inc., Watertown, MA, U.S.A.).

Cloning and Identification of the DNA Fragment

The synthesized DNA fragment *SQ4* and vector pET-32a(+) were digested with *Eco*RI and *Nco*I for 2 h at 37°C. Ligation and transformation into *E. coli* strain DH5α were accomplished by standard protocols [19]. The recombinant expression vector pET-SQ4 was confirmed by DNA sequencing with a Big Dye teminator kit and an Applied Biosystems 377 DNA sequencer.

Expression and Purification of the Recombinant Fusion Protein

The vector pET-SQ4 was isolated from DH5α cells and transformed into the *E. coli* expression host strain BL21(DE3) according to Novagen protocol. The transformed strain was incubated in Luria Broth (LB) medium at 37°C until the OD_{600} reached 0.5, and IPTG (isopropyl-β-D-thiogalactopyranoside) was added for a final concentration of 1 mM to induce protein expression at 37°C for 4 h. Cells were harvested and analyzed by SDS-PAGE. The purified fusion protein was obtained according to the method described by Huang *et al.* [8] and Son *et al.* [20].

Preparation and Purification of the Polyclonal Antibody

A New Zealand white rabbit was used to obtain antiserum against the fusion protein. Five-hundred µg of fusion protein were emulsified in Freund's complete adjuvant and administered subcutaneously at multiple sites on a healthy rabbit. The rabbit was given a booster injection after one month, then one injection every week for three weeks. Ten days after the last booster, the rabbit was bled and antiserum was collected. *E. coli* strain BL21(DE3) with blank vector pET-32a(+) was incubated in LB medium at 37°C, the protein expression induced, and the cells were harvested according to the procedure described above. The antiserum was purified from acetone-dried precipitate to remove the nonspecific antibody, according to the standard procedure [19].

Specificity Analysis of the Polyclonal Antibody

The specificity of the purified polyclonal antibody was confirmed by Western blot analysis. Total soluble protein was isolated from tobacco leaves; 150 µg of protein was transferred to the membrane and blotted according to the procedure of Huang *et al.* [8]. The cell extract of *E. coli* BL21 and *E. coli* BL21 transformed with pET-SQ4 was obtained according to the procedure of Chang and Kwak [2]. Rabbit antiserum (diluted 1:300 in 5% DM/PBS) (phosphate-buffer saline with 5% powdered lipid-free milk) was used as the first antibody, and an alkaline phosphatase conjugated anti-rabbit IgG goat antiserum (Promega, USA) (diluted 1:5000 in the above buffer with 5% powdered lipid-free milk) as the second antibody.

Expression Characteristics of Squalene Synthase at Different Stages of Development of *G. lucidum*

The primordium, small fruiting body, and mycelium incubated for 10, 12, 14, 16, 18, and 20 days were collected. Mycelium from liquid culture was handled according to the methods of Zhong [26]. Total soluble proteins were extracted according to the procedure described above, and 150 µg of total soluble protein from each sample were loaded into the wells of 12% SDS-PAGE and analyzed by Western blot as described above.

RESULTS

Synthesis of SQ4 and Construction of its Expression Vector

According to the preferred codon usage of *E. coli*, sense and antisense oiligonucleotides were designed and synthesized. After annealing, the *SQ4* fragment was synthesized using Pfu DNA polymerase systems. The synthesized *SQ4* DNA

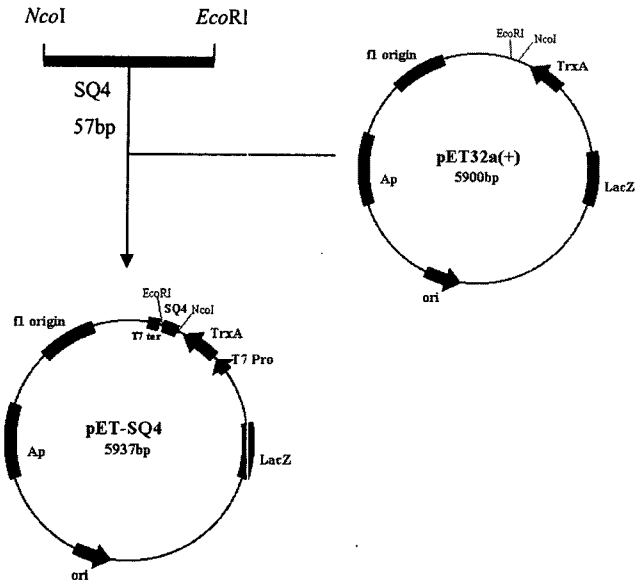

Fig. 1. Construction of recombinant expression plasmid pET-SQ4.

fragment was digested with *Nco*I and *Eco*RI restriction enzymes, and the digested fragments were ligated into pET-32a(+) vector digested with the *Nco*I and *Eco*RI restriction enzymes, producing the recombinant plasmid pET-SQ4 (Fig. 1), with the sequence shown in Fig. 2.

Expression of the Recombinant Protein

The *E. coli* expression host strain BL21(DE3) was transformed with the expression vector pET-SQ4. The result of SDS electrophoresis showed that the recombinant fusion protein with a molecular mass of 20.9 kDa was highly expressed in the transformed bacterium. Without induction by IPTG, recombinant protein could not be detected. Moreover, the recombinant protein migrated more slowly than the protein expressed in the pET-32a(+) without the *SQ4* (Fig. 3).

Production of Polyclonal Antibody and its Specificity Analysis

A polyclonal antibody was obtained from the serum of a New Zealand rabbit inoculated with purified recombinant fusion protein. To remove the nonspecific antibody, an acetone-dried precipitate from the *E. coli* containing pET-

*Nco*I restriction site
(DNA sequence)AAAACCATG GGC AGC ATG GGC CTG TTT CTG CAG AAA ACC AAC ATC
(amino acid sequence) S M G L F L Q K T N I
*Eco*RI restriction site
ATC CGT GAT TAT AAC GAA GAT CTG *GAATTCTTTT*
I R D Y N E D L

Fig. 2. Nucleotide and deduced amino acid sequences of the designed SQ4.

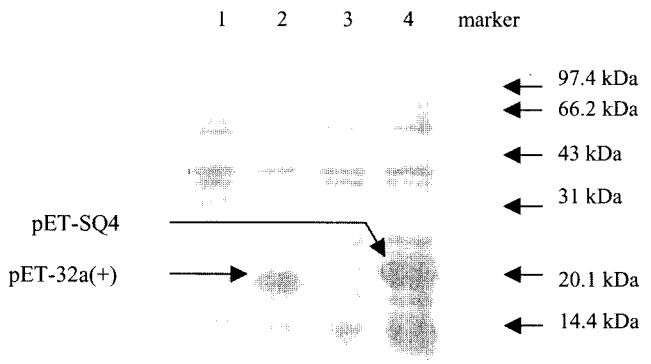

Fig. 3. SDS-PAGE analysis of the recombinant SQ4 fusion protein expressed in *E. coli.*
1, Lysate of bacteria with pET-32a(+) without IPTG induction. 2, Lysate of bacteria with pET-32a(+) after IPTG induction. 3, Lysate of bacteria with pET-SQ4 without IPTG induction. 4, Lysate of bacteria with pET-SQ4 after IPTG induction. 5, Molecular weight (mw) marker; fragments: 97.4, 66.2, 43, 31, 20.1, and 14.4 kD.

32a(+) induced by IPTG was prepared. Western blotting was carried out to confirm the specificity of this antibody using total soluble protein extracted from tobacco leaves. The assay detected an expected protein band with a molecular mass of 47.1 kDa in tobacco extract, identical to the result reported by Hanley and Chappell [5], indicating that this polyclonal antibody specifically reacts to squalene synthase (Fig. 4A). It also showed a positive band with a molecular mass of about 20.9 kDa in the cell extract of *E. coli* BL21 transformed with pET-SQ4, and no signal in the cell extract of *E. coli.* BL21, suggesting that this

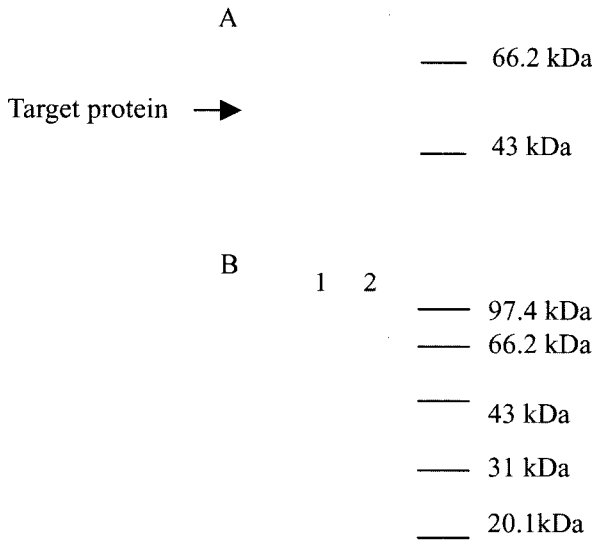

Fig. 4. Western blot analysis of squalene synthase.
A. Analysis of squalene synthase in *Nicotiana tabacum.* B. Analysis of the fusion protein in *E. coli* BL21. 1, Cell extract of *E. coli* BL21. 2, Cell extract of *E. coli* BL21 transformed with pET-SQ4.

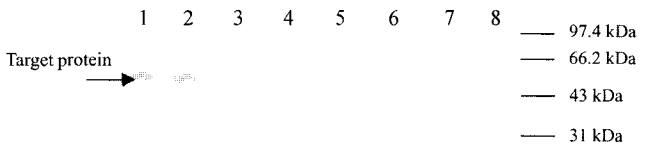

Fig. 5. Western blot analysis of squalene synthase at different stages of development in *Ganoderma lucidum*.
1, Lysate of small fruiting body. 2, Lysate of primordium. 3, Lysate of mycelium after 20 days of incubation. 4, Lysate of mycelium after 18 days of incubation. 5, Lysate of mycelium after 16 days of incubation. 6, Lysate of mycelium after 14 days of incubation. 7, Lysate of mycelium after 12 days of incubation. 8, Lysate of mycelium after 10 days of incubation.

polyclonal antibody could specifically react with the fusion protein (Fig. 4B).

Analysis of Expression Characteristics of Squalene Synthase in *G. lucidum* at Different Stages of Development

Mycelium was cultured at 28°C for 10, 12, 14, 16, 18, and 20 days, and primordium and small fruiting body were collected. Total soluble proteins were extracted from all materials. Western blot analysis showed that there is a positive band with a molecular weight of about 48 kDa in *G. lucidum*. Squalene synthase reactivity could not be detected in mycelium incubated for 10 to 16 days, but it could be detected after 18 days. However, the strength of the reaction was much weaker than those of the primordium and the small fruiting body (Fig. 5), suggesting that squalene synthase was highly expressed in primordium and small fruiting body.

DISCUSSION

The nucleotide sequences of the gene encoding squalene synthase in yeast [9, 25], humans [10], tobacco [5], and *Arabidopsis thaliana* [22] have been published, but little is known about this enzyme in *G. lucidum*. It was assumed that squalene synthase in *G. lucidum* has the same conserved region as in the published sequences. To analyze the mechanism of triterpene biosynthesis in *G. lucidum*, a DNA fragment encoding the fourth conserved domain of squalene synthase was synthesized and overexpressed in *E. coli* as fusion protein. A specific polyclonal antibody was obtained from a rabbit immunized with the purified fusion protein. Thereafter, the expression level of squalene synthase in this medical mushroom at different development stages was investigated.

The expression level of squalene synthase in *G. lucidum* varied at different stages of development. In the early stage, little squalene synthase was detected in mycelium after incubation. It was concluded that the low expression level of this enzyme is correlated with low content of triterpenes. The expression level increased dramatically from primordium to small fruiting body. The high content of the enzyme might account for activation of the synthesis of triterpenes. Hirotani *et al.* [6] observed that the content of triterpenes was much lower in the mycelium, and higher in the fruiting body of *G. lucidum*. With the construction of full-length cDNA encoding squalene synthase in *G. lucidum*, further study is expected to yield insight on the mechanism of the triterpene synthesis.

Acknowledgments

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