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Research Article

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Molecular Characterization of a Squalene Synthase Gene from *Phellinus igniarius*

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Abstract

According to the conserved sequence of SQS（squalene synthase） from NCBI database, designed primers and amplified SQS gene fragment with RT-PCR product from Phellinus igniarius total RNA. Then obtained full length SQS gene using the RACE technology. Analyzed the amino acid sequences, and constructed phylogenetic tree. The SQS cDNA was constructed into the prokaryotic expression vector pET22b and expressed in E.coli BL21(DE3). SDS-PAGE Analyzed protein expression. Lay the foundation for further analysis of the structure and function of squalene synthase.

Keywords: squalene synthase RT-PCR expression

INTRODUCTION

SQS (Squalene synthase) , a microsomal enzyme has an important role in the biosynthesis of sterols and triterpenoids, it is key enzyme in the formation of Squalene - sterol and trisomy compounds. High expression can promote synthes of triterpenoids. Because of its important role in the isoprenoid pathway, it become the current research focus.

SQS (squalene synthase) is located on the endoplasmic reticulum, catalyzes the two molecules of Farnesyl pyrophosphate alkenyl to form squalene, by squalene epoxidase (SE) and squalene cyclase (SC) [1,2]. Squalene is catalyzed to Synthesize triterpenoids, sterols, cholesterol, etc. as a key enzyme in synthesis pathway of triterpenoids[3], now SQS gene were cloned from *Artemisia annua*, *Psammosilene tunicoides*, Glycyrrhiza uralensis, *Arabidopsis thaliana* and other plants[4,5].

Triterpenoids is the more common type compound in *Phellinus igniarius*, its medicinal value in *Phellinus igniarius* is currently a research focus[6,7], *Phellinus igniarius* is currently the first efficiency in the field of biological treatment of cancer by internationally recognized, because of its unique anti-cancer effect, It has been a hot topic for

scholars, in addition to the pharmacological effects in traditional Chinese medicine theory, *Phellinus igniarius* also has anti-cancer, anti-tumor, immunomodulatory, liver health[8,9], It has great significance for the Further research and development of *Phellinus igniarius.*

EXPERIMENTAL SECTION

Strains, plasmids and culture conditions

Phellinus igniarius was presented from China University of Petroleum, Cultured on PDA medium, 28 ℃, 140rpm for 7 days. E. coli DH 5α, BL21 (DE3) were stored by our laboratory. restriction enzymes, DNA polymerase, reverse transcription kit, Trizol reagents were purchased from Takara Bio Company, Plasmid pMD18-T was purchased from Promega Corporation, the prokaryotic expression vector pET22b was stored by our laboratory, DNA sequenced by Sangon Biotech.

Total RNA extraction and first-strand cDNA synthesis

Take *Phellinus igniarius* cultured for 7d, cells were collected by centrifugation, water was dried by sterile filter paper, ground in liquid nitrogen and then extracted RNA by Trizol method, and finally eluted RNA adding DEPC-H_2O, electrophoretic analysis of RNA quality. Take the total RNA 5μg and Oligo-dT primer in a cDNA synthesis mix Buffer, and after 70 ℃ denaturation for 5min, added dNTP and RNA reverse transcriptase, and then incubated at 37 ℃ for 1h, obtained cDNA first strand as a template for PCR reaction.

Primer design and RT-PCR

According to the published gene sequences of SQS, we designed a pair of degenerate primers sequence, as follows: P1: 5'-TAYTGYCAYTAYGTNGC-3'; P2:5'-ACYTGNGDATNGCRCARAA-3 '. by RACE to obtain two ends of SQS cDNA sequence, Specific PCR primers used to amplify the SQS sequence: P3:5'-ATGGCGACCACTC GCAACTC-3';P4:5'-CTACTCCTGGGTGATGTAATA-3'.

Bioinformatics analysis of SQS gene

Sequenced the full-length cDNA sequence, analyzed amino acids sequence of protein, predicted protein molecular weight and isoelectric point. Download SQS protein sequence of other species from NCBI to construct phylogenetic tree based on amino acids, Analyzed the evolutionary status of SQS in *Phellinus igniarius.*

Construction of Prokaryotic expression vector

According sequenced *Phellinus igniarius* SQS cDNA to design primer, upstream primer: 5'-TATCATATGGCGACCACTCGCAACTCTTAC-3' with the *Nde*I recognition site (underlined) Downstream primer: 5'-GCCAAGCTTCTCCTGGGTGATGTAATAC-3' with the *Hin*dⅢ recognition site (underlined) .Amplified full-length sequence of the coding region of SQS, and then connected to the fusion expression vector pET22b after purification, constructed fusion expression vector pET22b-SQS, transform competent *E. coli* BL21, PCR screened recombinant, sequencing to identify the gene sequence inserted.

Protein expression and analysis with SDS-PAGE

BL21 strains containing recombinant plasmid pET22b-SQS were cultured in LB medium with Ampicillin, 37 ℃ shaked for about 12h, and then expanded at 1: 100, when OD600nm reach 0.4-0.6, IPTG was added to a final concentration of 0.2mmol / L ,and then cultured at 28℃for about 4h. Cell were harvested by centrifugation, and SDS-PAGE to detect the expression.

RESULTS AND DISCUSSION

Sequence analysis of the SQS gene

According to SQS sequences of other organisms, designed degenerate primers to obtain conserved regions of SQS, sequenced to get complete information of this sequence, and then redesigned primers to amplify the complete sequence using RACE-PCR technology (Fig.1a), according to sequencing results obtained Amino acid composition (Fig.1b). Predicted protein function using the website (http://smart.embl-heidelberg.de/), there is no signal peptide region and it has a functional area.

Phylogenetic analysis of PiTPS

According to NCBI database, collected SQS protein sequences of other organisms,Phylogenetic tree was constructed based on the SQS amino acid sequence previously collected with SQS sequence *phellinus igniarius* (Fig.2), Further analyzed the evolution status and relationship of SQS with other organisms.

SDS-PAGE assay of the recombinant PiTPS protein

The engineering strain was cultured overnight, Induced culture after grown to logarithmic phase. Cells were harvested by centrifugation, SDS-PAGE analysis of protein expression, there is a specific band was found at 42.19kDa of engineering strain, achieved a prokaryotic expression of SQS(Fig. 3)

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Fig. 1 (a)Nucleotide sequences of SQS gene. The letters in shadow indicated the start codon (ATG) and asterisk indicated the stop codon (TAG). (b) Deduced amino acid total 387 amino acids

(a)

ATGGCGACCACTCGCAACTCTTACGACGTTCTCATTGTTGGCGGTGGTGTCGCCGGGTGTGCTCTTGCGT
ACAGTCTCTCGACCATTACCTCCAAAGCACGTCCAAAGCAACTCCGTATCGGTCTCATTGAACGCTCCTT
TGCAGAACCAGACCGCATTGTCGGCGAACTTCTTCAGCCTGGCGGGTGTAATGCACTTCGCAAACTTGG
ATTAGGCGATTGCCTAGAAGGTATAGACGCAGCGGCTGTGCGTGGTTATTGCGTTGTCAACGACGGGAA
GCAAGTCCATATTCCATATCCTAACGGCCAAGAGGGACGGGCTTTCCATCATGGCAAATTTATCATGGCA
TTGAGGAAGAAGGCTCTCACGGCTCCCGGTGTCGAAGGAATTGAAGCTACAGTCACGTCGCTCCTTAC
CGCAGACGGCGAGCACCGGGTCTACGGTGTTTGCGCGACGCGTAAGAACTCTGGGACTGAAACGAAG
GAGAACTTCTATGCACCCCTCACGTTCGTTGCCGATGGCTGCTTCTCCAATTTCCGAGCAGAGGTCTTGA
GCGAAGCCTTTGAGAAACCCGTCACACGCAGCTACTTCGTCGGTGCGATTCTCAAGGACGTAAAATTGC
CGATCGACAAACATGGAACCGTCGCTCTTGTCCGTGGGTCTGGTCCTGTGCTGCTGTACCAAATTGCCG
AACATGACACACGTATACTCATCGATGTTAAAGCACCCTTGCCATCTGACCTCAAACAATTCGTACTGGA
CAAGGTTTTGCCCGAACTCCCAGAAAGTGTTCAGCCGGCTCTCGTAGATGCTCTCAACAGGGAGCGGCT
ACGTCGTATGCCCAATTCCTTCCTTCCTCCCATTGAGCAAGGCGGAGAGCAGTCCAAGGAGGGTGCTAT
TCTTGTTGGCGACTCGTGGAATATGCGTCATCCCCTTACCGGAGGTGGAATGACCGTCGCCTTCAATGAT
ATTGTCATACTTACGGATCTTCTTCGCACTGTACCGAACTTCGAGGACTGGGCATGCGTATCGCAGATCC
TTCATGCATGGCATTGGTCGCGTAAGCCGCTTGGCTCGACGATCAATATTCTTAGTATCGCGCTTTACGAC
CTCTTCGGCGCCGAGGGTCTGTCACATTCTCTGTATTACATCACCCAGGAGTAG

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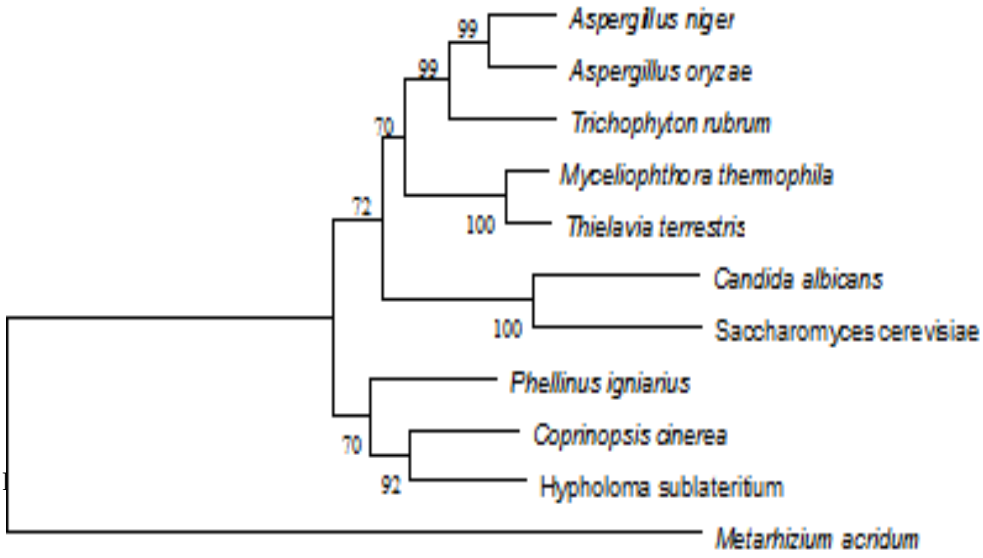
(b)

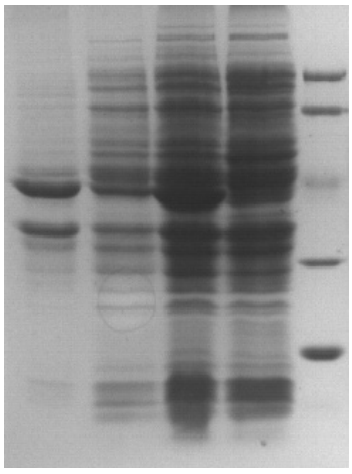
MATTRNSYDVLIVGGGVAGCALAYSLSTITSKARPKQLRIGLIERSFAEPDRIVGELLQPGGCNALRKLGLGD
CLEGIDAAAVRGYCVVNDGKQVHIPYPNGQEGRAFHHGKFIMALRKKALTAPGVEGIEATVTSLLTADGEH
RVYGVCATRKNSGTETKENFYAPLTFVADGCFSNFRAEVLSEAFEKPVTRSYFVGAILKDVKLPIDKHGTVA

LVRGSGPVLLYQIAEHDTRILIDVKAPLPSDLKQFVLDKVLPELPESVQPALVDALNRERLRRMPNSFLPPIEQ
GGEQSKEGAILVGDSWNMRHPLTGGGMTVAFNDIVILTDLLRTVPNFEDWACVSQILHAWHWSRKPLGSTI
NILSIALYDLFGAEGLSHSLYYITQE

Fig. 2 Schematic representation of SQS protein, drawn to corresponding scales as in the fig

Fig. 2 Phylogenetic analysis of PiSQS showing relationship with other known SQS. Alignment of amino acid sequences with CLUSTALW, consensus Neighbor-Joining tree with MEGA5.0





M :standard protein molecular weight; 1:crude enzyme from BL21 at 28 ℃for 4h;
2:crude enzyme from BL21 (DE3)/ pET22b-SQS induced by IPTG (0.2mMol) at 28℃ for 4 h; 3: Precipitation of crude enzyme;4: Supernatant of crude enzyme

DISCUSSION

This study successfully cloned squalene synthase gene which is the key enzyme in triterpenoid biosynthesis pathway[10-12], sequenced the full-length cDNA sequences and analyzed by bioinformatics. Bioinformatics analysis is an effective means to predict gene function. It provides a theoretical basis for further design and by verification experiments.

As we all knows the similarity of plants squalene synthase sequence are higher. SQS of *Phellinus igniarius* encodes 387 amino acids, the molecular weight is 42.19 kD, and pI is 6.56. Another feature of SQS gene, which the similarity of amino acid sequence between plant、 animals and fungi have low homology, about 35% to 40%. but higher in the plants (tobacco and *Arabidopsis thaliana*), in animals (human and mouse) high similarity, and fungi showed a trend of multi-differentiation[13,14].

We use advantageous combination with the fusion expression vector pET22b and BL21 (DE3) strain, and obtained soluble and intact protein of SQS. pET22b carry an N-terminal pelB signal peptide sequence, the target protein can be localized in the periplasmic cavity, meanwhile the vector contains a C-terminal His-tag for purification of proteins. *Escherichia coli* BL21 (DE3) strains with T7 RNA polymerase gene can be induced by IPTG, andstrains lack the lon protease and outer membrane proteases ompT can prevent the degradation of Protein. All these can help to express the target protein effectively.

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