Expression, Purification, Characteristics and Homology Modeling of the HMGS from *Streptococcus pneumoniae*[1]

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Objective To understand the molecular basis for a potential reaction mechanism and develop novel antibiotics with homology modeling for 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase (HMGS). **Methods** The genetic engineering technology and the composer module of SYBYL7.0 program were used, while the HMGS three-dimensional structure was analyzed by homology modeling. **Results** The mvaS gene was cloned from *Streptococcus pneumoniae* and overexpressed in *Escherichia coli* from a pET28 vector. The expressed enzyme (about 46 kDa) was purified by affinity chromatography with a specific activity of 3.24 μmol/min/mg. Optimal conditions were pH 9.75 and 10 mmol/L $MgCl_2$ at 37 ℃. The V_{max} and K_m were 4.69 μmol/min/mg and 213 μmol/L respectively. The 3D model of *S.pneumoniae* HMGS was established based on structure template of HMGS of *Enterococcus faecalis*. **Conclusion** The structure of HMGS will facilitate the structure-based design of alternative drugs to cholesterol-lowering therapies or to novel antibiotics to the Gram-positive cocci, whereas the recombinant HMGS will prove useful for drug development against a different enzyme in the mevalonate pathway.

Key words: *Streptococcus pneumoniae*; HMG-CoA synthase; Analysis of dynamics; Homology modeling

INTRODUCTION

Streptococcus pneumoniae, one of pathogenic bacteria causing bacterial meningitis, often causes serious nervous system sequelae with higher fatality rate. In recent years, *S. pneumoniae* has acquired resistance to penicillin and macrolides antibiotics and has an increasing trend year after year because of the environmental pollution and their usage or in-appropriate usage of large quantity in clinical treatment, while the multi-drug resistance of other antibiotics (such as fluoroquinolones) emerges[1-3]. It has become a cause of invasive diseases in many countries, and antibiotic treatment has become less effective[4]. Therefore, it is essential to seek for new target in organism's metabolism and exploit new antibiotics with homology modeling.

In organisms, isopentenyl diphosphate (IPP) plays some important functions in photosynthesis, respiration, regulation of growth and development. Mevalonic acid serving as the precursor for the synthesis of cholesterol, the side chain of ubiquinone, dolichols, the isopentenyl adenine of certain tRNAs and other isoprenoids, is generated in two major pathways. One is non-mevalonate pathway found in most eubacteria, and the other is mevalonate pathway (Fig. 1) found in animal cells and a number of pathogenic bacteria[5]. Three enzymes participate in the mevalonic acid biosynthesis. That is: (1) acetoacety-CoA thiolase (AACT, EC 2.3.1.9) catalyzing a Claisen-type condensation of two acetyl-CoA molecules to form the intermediate acetoacetyl CoA (AcAc-CoA)[6-7]; (2) 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS, EC 4.1.3.5), catalyzing a aldol condensation of one molecule of AcAc-CoA with acetyl-CoA to form one molecule of S-HMG-CoA[8–10]; (3) HMGCoA reductase (HMGR, EC 1.1.1.34), a NADPH-dependent and membrane-bound enzyme, which is generally considered to be a key enzyme catalyzing the regulatory step in the mevalonic acid generation from HMG-CoA[11]. HMG-CoA synthase plays an essential role in this

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pathway.

HMG-CoA reductase catalyzing the rate-limiting step in IPP biosynthesis was successfully cloned and expressed in our laboratory. HMG-CoA synthase is the key enzyme preceding HMG-CoA reductase in this pathway (Fig. 1) catalyzing the condensation of acetyl-CoA and AcAc-CoA to form HMG-CoA. This paper reported the cloning, back mutation, expression, purification, and kinetic characteristics of HMGS, and designed the 3D model of *S. pneumoniae* HMGS through three-dimensional structure analysis using homology modeling, which may provide the molecular basis for a potential reaction mechanism and provides insight to aid rational drug design. In addition, there are two classes of HMG-CoA reductase that differ both with respect to their construction and their inhibition characteristics[12], and there may be significant differences between the bacterial and human forms of other enzymes in the pathway. The enzymes of the mevalonate pathway may therefore represent potential targets for the development of new antibiotics.

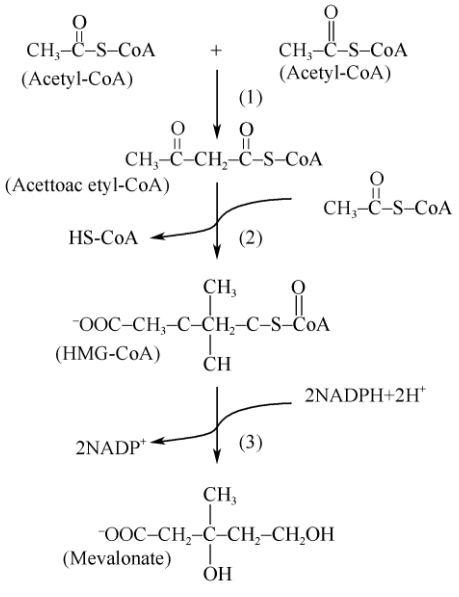

FIG. 1. The mevalonate pathway (1)Acetyl-CoA acetyltransferase; (2)HMG-CoA synthase; (3)HMG-CoA reductase.

MATERIALS AND METHODS

Materials

Escherichia coli DH5α, BL21, and pET-28 vectors were kept in our laboratory. HisTrap™ FF crude 1 mL column were purchased from GE Healthcare Co. RNase, restriction enzymes, Taq DNA polymerase, T4 DNA ligase, isopropyl-β-D-thiogalactopyranoside (IPTG), and DNA molecular weight markers were purchased from Takara. KOD-Plus-Ver.2 was from TOYOBO Co. and Dpn Ⅰ was from NEW ENGLAND BioLabs Co. Acetyl-CoA and AcAc-CoA were purchased from Sigma. DTT, HEPES, PMSF were purchased from Bio-Rad. *S. pneumoniae* strains were from Wuhan University, *S. pneumoniae* was seeded on nutrition agar with added 10% sheep blood. Inoculated media were incubated at 37 ℃ for 18-20 h in an atmosphere of 5% carbon dioxide[13].

Construction of Recombinant Vector

Genomic DNA was prepared from *S. pneumoniae* with the standard method[14]. The fragments of the *mvaS* gene were amplified using the *S. pneumoniae* genomic DNA as template, and the primers which embed flanking *Eco*RI and *Not*I restriction sites designed based on the *S. pneumoniae* genome sequence (AF290098), P1 (5′-GGAATT-CATGAA TGATAAAACAGAGGTA AATAT-3′) and P2 (5′-ATAGTTTAGCGGCC GCAACTTATCTTCATT ATTTTTCAACC-3′). Reaction condition was as follows: pre-denaturalization 94 ℃ 5 min, and then 94 ℃ 30 s, 50 ℃ 30 s, 72 ℃ 1 min for 30 cycles, finally 72 ℃ 10 min. PCR products were performed following the instructions of the AxyPrep™ DNA Gel Extraction Kit. The amplified DNA was inserted into pET28a expression vectors containing a His-tag encoding sequence.

Back Mutation

One nucleotide inserted fragment was detected to have been mutated by DNA sequencing from pET-HMGS. The primers which embed the correct nucleotide in middle site were designed based on the *S. pneumoniae* genome sequence (AF290098) according to the demand of site mutation, P1 (5′-CTCTATTTTAACTGAGCAAGAAAGACAAGAA GTTGTCATT G-3′) and P2 (5′-CAATGACCATGTC AACTTCTTGTC TTTCTTGCTC AGTTAAAATA-GAG-3′) (the corrected nucleotide was underlined). Using KOD-Plus-Ver.2, PCR was conducted as follows: pre-denaturalization 94 ℃ 5 min, and then 94 ℃ 1 min, 55 ℃ 1 min, 68 ℃ 7 min for 16 cycles, finally 16 ℃ hold. The product of PCR was treated for 3 h with Dpn Ⅰ to remove template DNA and the mutated DNA was transformed into *E. coli* DH5α competent cells.

Recombinant HMGSm Expression and Purification

The recombinant plasmid pET28-HMGRm was

expressed as fusion proteins in *E. coli* BL21 (DE3) in LB media, containing 50 μg/mL kanamycin. When the cells grew to mid-log phase, the protein expression was induced by adding IPTG to a final concentration of 0.4 mmol/L. After 4 h incubation at 18 ℃, bacteria were harvested by centrifugation at 8000 rpm for 10 min at 4 ℃. The deposit resuspended in ice-cold buffer A (containing 300 mmol/L NaCl, 1 mmol/L PMSF，1 mmol/L DTT, 20 mmol/L HEPES, pH 8.0) was sonicated condition, and the supernatant were collected and analyzed by SDS-PAGE. Purification of the recombinant HMGSm was accomplished using HisTrap^{TM} FF crude 1 mL column. The cell supernatant fraction was loaded onto a Ni^{2+} metal HisTrap^{TM} FF Column equilibrated with buffer A. The column was washed with 5 column volumes of buffer A containing 10 mmol/L imidazole. The enzyme was eluted from the column in 1 mL fractions with 3 mL buffer A containing 500 mmol/L imidazole. Protein concentration was detected by NanoDrop Sectrophotometer (ND-1000, Gene Co. Limited).

Assay for HMGSm Activity

Activity of HMGS was analyzed with the S-3100 UV-Vis spectrophotometer (S-3100，SCINCO Co.) at 37 ℃ based on 300 nm of the oxidation of AcAc-CoA. Based on a method developed for *Enterococcus faecalis*[15], standard assay conditions (in a final volume of 220 μL) for each reaction were as follows: 500 μmol/L acetyl-CoA，20 μmol/L AcAc-CoA, 5 mmol/L $MgCl_2$, 50 mmol/L Tris (pH 9.75). Reaction mixtures were first monitored to detect acetyl-CoA independent disappearance of AcAc-CoA. For all reactions, 1 unit of HMGS was defined as the amount of enzyme which represented the disappearance of 1 μmol AcAc-CoA per min. The kinetic parameters V_{max} and K_m were determined using dual-reciprocal plots.

Homology Modeling

The HMGS sequence was submitted to the SWISS-MODEL sever (Automated Comparative Protein Modeling Sever, Version 3.5, Glaxo Wellcome Experiment Research, Geneva, Switzerland) for comparative structural modeling[16-17]. The homology model of the HMGS of *S. pneumoniae* was built based on sequence alignment. The template sequence of 1X9E[18] which was selected as template for homology modeling has more than 53% identity with the target sequence[19]. All hydrogen atoms were subsequently added to the unoccupied valence of heavy atoms at the neutral state using the biopolymer module of SYBYL7.0 program package (http://www.tripos.com)[20]. Further energy minimization was performed to remove the geometric restraints before the model was constructed.

RESULTS

Cloning and Back Mutation of HMGS

A 1.3 kb fragment of *mvaS* gene was amplified by PCR, and the identification of the recombinant expression plasmid pET-HMGS was confirmed by *Eco*RI/*Not*I digestion (Fig. 2A). DNA sequencing assay showed that a termination codon occurred after one nucleotide of the *mvaS* gene was mutated (Fig. 3). The fragments of the *mvaS* gene were amplified to conduct back mutation, using pET-HMGS as template and using the site mutation primers which brought the correct nucleotide instead of the mutated one. The directions of *mvaS* in the recombinant expression plasmid pET-HMGSm was identified and confirmed by *Eco*RI/*Not*I and *Eco*RI/*Hind*III digestion (Fig. 2B) and was further confirmed by DNA sequencing.

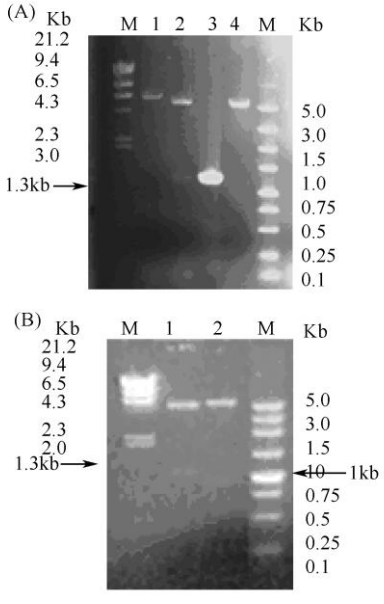

FIG. 2. Identification of recombinant expression plasmid. (A) Identification of the recombinant plasmid pET-HMGS, lane 1: pET-HMGS/*Eco*RI; lane 2: pET-HMGS/*Eco*RI &*Not*I; lane 3: PCR product of HMGS; lane 4: pET/*Eco*RI; M: DNA Marker. (B) **I**dentification of recombinant expression plasmid pET-HMGSm, M: DNA Marker; lane 1: pET-HMGSm/*Eco*RI&*Not*I; lane 2: pET/*Eco*RI &*Hin*d III.

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1    ATGAATGATAAAACAGAGGTAAATATGACAATCGGTATTGATAAGATTGGTTTTGCGACCAGTCAATATGTCTTGAAA
1     M  N  D  K  T  E  V  N  M  T  I  G  I  D  K  I  G  F  A  T  S  Q  Y  V  L  K
79   TTACAAGACTTAGCAGAAGCGAGGGGAATTGACCCTGAAAAATTAAGTAAAGGACTCTTACTCAAGGAATTGAGTATT
27    L  Q  D  L  A  E  A  R  G  I  D  P  E  K  L  S  K  G  L  L  L  K  E  L  S  I
157  GCGCCCCTAACTGAGGACATCGTGACCTTGGCGGCCAGTGCTAGTGACTCTATTTTAACTGAGCAAGAATGACAAGAA
53    A  P  L  T  E  D  I  V  T  L  A  A  S  A  S  D  S  I  L  T  E  Q  E  *  Q  E
235  GTTGACATGGTCATTGTGGCGACCGAGTCAGGAATTGACCAGAGTAAGGCTGCGGCCGTCTTTGTGCATGGCTTGCTG
79    V  D  M  V  I  V  A  T  E  S  G  I  D  Q  S  K  A  A  A  V  F  V  H  G  L  L
313  GGCATCCAGCCCTTTGCTCGTAGTTTCGAGATTAAAGAAGCCTGCTATGGGGCGACTGCTGCCCTCCATTATGCCAAA
105   G  I  Q  P  F  A  R  S  F  E  I  K  E  A  C  Y  G  A  T  A  A  L  H  Y  A  K
391  TTGCATGTGGAAAATTCTCCGGAGTCCAAGGTCTTGGTCATTGCAAGTGATATTGCCAAATACGGTATTGAAACTCCG
131   L  H  V  E  N  S  P  E  S  K  V  L  V  I  A  S  D  I  A  K  Y  G  I  E  T  P
469  GGAGAACCCACTCAGGGTGCCGGAAGTGTGGCTATGTTGATTACACAAAATCCACGCATGATGGCCTTTAATAATGAC
157   G  E  P  T  Q  G  A  G  S  V  A  M  L  I  T  Q  N  P  R  M  M  A  F  N  N  D
547  AATGTAGCTCAGACCCGTGACATCATGGATTTCTGGCGACCAAATTACTCGACAACTCCTTATGTAAATGGTGTCTAT
183   N  V  A  Q  T  R  D  I  M  D  F  W  R  P  N  Y  S  T  T  P  Y  V  N  G  V  Y
625  TCTACCCAACAATACTTGGATAGTTTGAAAACGACTTGGCTTGAATATCAAAAACGCTACCAGCTTACTTTGGATGAT
209   S  T  Q  Q  Y  L  D  S  L  K  T  T  W  L  E  Y  Q  K  R  Y  Q  L  T  L  D  D
703  TTTGCGGCTGTTTGTTTCCACTTGCCTTATCCTAAATTAGCGCTAAAAGGCTTGAAAAAAATCATGGATAAGAGCCTG
235   F  A  A  V  C  F  H  L  P  Y  P  K  L  A  L  K  G  L  K  K  I  M  D  K  S  L
781  CCTCAAGAGAAAAAAGACCTCTTACAAAAGCATTTTGACCAGTCTATTCTCTACAGTCAAAAGGTGGGGAATATCTAC
261   P  Q  E  K  K  D  L  L  Q  K  H  F  D  Q  S  I  L  Y  S  Q  K  V  G  N  I  Y
859  ACAGGTTCACTTTTCCTTGGACTTTTGTCTCTCTTGGAAAATACAGATAGCTTGAAAGCTGGGGATAAAATCGCCCTT
287   T  G  S  L  F  L  G  L  L  S  L  L  E  N  T  D  S  L  K  A  G  D  K  I  A  L
937  TATAGTTACGGAAGTGGAGCTGTGGCTGAGTTCTTCAGTGGTGAATTGGTTGAAGGATATGAAGCTTATTTGGATAAA
313   Y  S  Y  G  S  G  A  V  A  E  F  F  S  G  E  L  V  E  G  Y  E  A  Y  L  D  K
1015 GACCGCTTGAACAAGCTCAACCAACGAACTGTCTTATCCGTTGCAGACTATGAAAAGGTCTTTTTTGAGGAAGTAAAC
339   D  R  L  N  K  L  N  Q  R  T  V  L  S  V  A  D  Y  E  K  V  F  F  E  E  V  N
1093 TTGGATGAAACAAACTCTGCCCAGTTTGCTGGCTATGAAAATCAAGATTTTGCCTTGGTTGAAATTCTCGACCACCAA
365   L  D  E  T  N  S  A  Q  F  A  G  Y  E  N  Q  D  F  A  L  V  E  I  L  D  H  Q
1171 CGCCGTTATAGCAAGGTTGAAAAATAA
391   R  R  Y  S  K  V  E  K  *
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FIG. 3. The HMGS sequence in pET-HMGS, the mutated nucleotide and its translated codon were underlined.

Expression and Purification of HMGSm

The recombinant expression vectors pET-HMGSm and pET-HMGS were transformed into *E.coli* BL21 and expressed with 0.4 mmol/L IPTG induction at 18 ℃. When the recombinant expression vector pET-HMGS with the termination codon was expressed, the specific protein band was only about 10 kDa (Fig. 4A); while the recombinant expression vector pET-HMGSm was transformed and the product analyzed by SDS-PAGE was expressed, which showed that a specific protein band appeared with a molecular weight of about 46 kDa (Fig. 4B). The specific activity increased to 3.24 μmol/min/mg after the soluble crude extract was purified by HisTrap™ FF crude 1 mL column (Fig. 4C).

Kinetic Characteristics of HMGSm

The activity of HMGSm from the recombinant HMGSm was detected with spectrophotometer based on the disappearance of the absorption at 300 nm of AcAc-CoA (Fig. 5). Optimal activity of the HMGSm expressed in *E. coli* was detected at pH 9.75 (Fig. 6A), approximately at 37 ℃ (Fig. 6B) and 10 mmol/L $MgCl_2$ (Fig. 6C). The V_{max} and K_m value were determined for the reaction of AcAc-CoA to HMG-CoA, which was 4.69 μmol/min/mg and 213 μmol/L respectively.

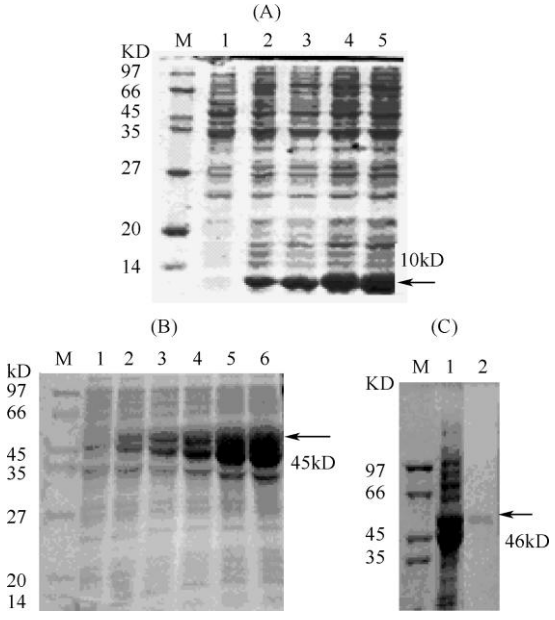

FIG. 4. Expression of the recombinant HMGS, HMGSm and purification of the recombinant HMGSm. (A) SDS-PAGE of the recombinant HMGS, M: Protein Marker; lane 1: no IPTG induction; lanes 2-5: induced for 2, 4, 6, 8 h. (B) SDS-PAGE of the recombinant HMGSm, M: Protein Marker; Lane 1: no IPTG induction; lanes 2-6: induced for 2, 4, 6, 8, 10 h. (C) Purification of the recombinant HMGSm, M: Protein Marker; lane 1: crude extract of *E.coli*; lane 2: the purified recombinant HMGSm protein.

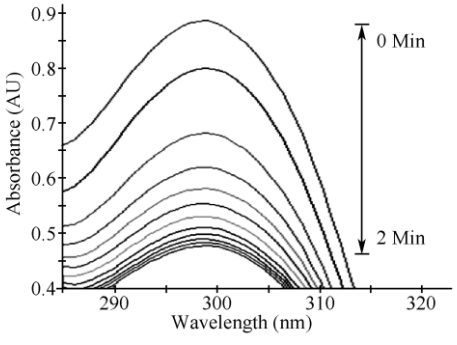

FIG. 5. Determination for the catalytic ability of HMGSm, the highest peak represented the initiation absorbance, the interval was 0.2 min, and the total time was 2 min.

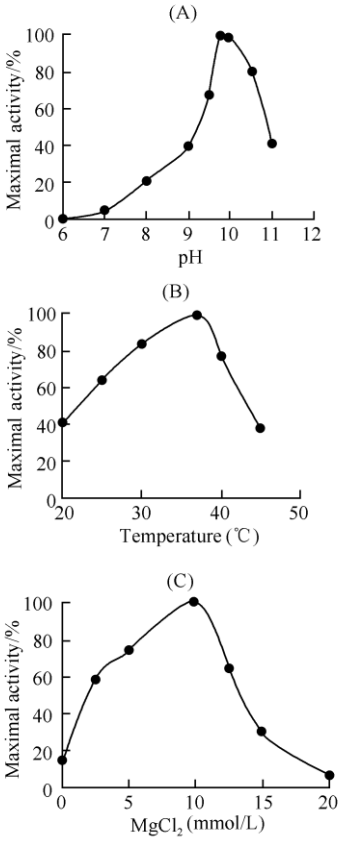

FIG. 6. Effect of pH, temperature, and $MgCl_2$ on HMGSm activity. (A)Effect of pH on activity; (B)Effect of temperature on activity; (C)Effect of $MgCl_2$ on activity.

Homology Modeling

In the absence of crystal structures, homology modeling was shown to be a valuable tool for gaining an insight into the interaction between substrates and proteins. In our project, the alignment displayed a strong similarity of the two sequences, and ensured the quality of the homology model. Further energy minimization was performed to remove the geometric restraints before the model was constructed. An accurate theory of three-dimensional (3D) structure of the HMGS of *S. pneumoniae* was built as shown in Fig. 7A, where β-strands was shown in purple and α-helices was shown in orange. Figure 7B shows the superposition of the target protein model with 1X9E. As expected, the overall conformation of the model was very similar to the template[21]. So an accurate 3D-structure of HMGS was obtained by homology model, and will be used for further study.

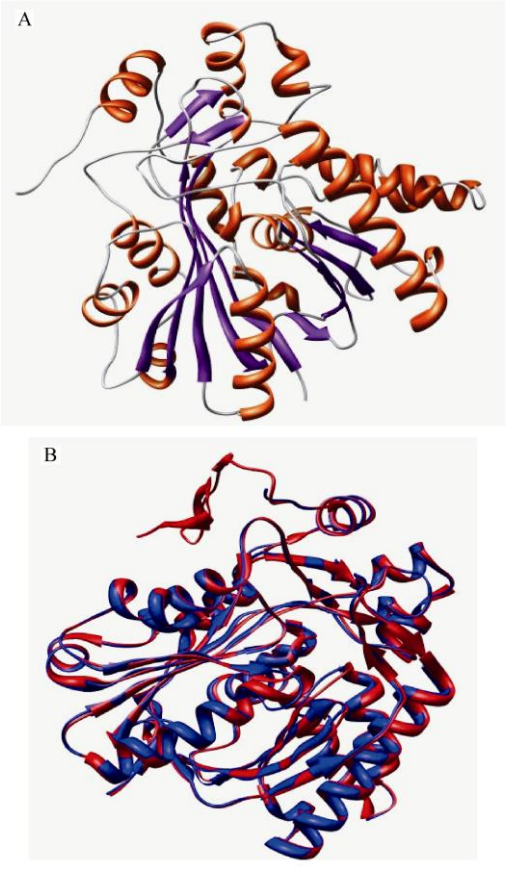

FIG. 7. Homology modeling of HMGS of *S. pneumoniae*. (A)The monomer HMGS with strands and helices, β-strands was shown in purple and α-helices was shown in orange; (B)The superposition of the target protein model with 1X9E, the blue was model and the red was 1X9E.

DISCUSSION

The enzymes of the mevalonate pathway of IPP biosynthesis represent potential targets for metabolic intervention. The mevalonate pathway is essential for the survival of *S. pneumoniae* and, by inference, for other gram-positive bacteria[22]. Molecular structures, biochemical properties and sequences comparisons have classified the living organisms into three domains: the eukaryotes, the eubacteria or true bacteria and the archaea. HMGR exists in all the three domains, including human. Although the mevalonate pathway is also essential for human subjects, there are two classes of HMG-CoA reductase that differ both with respect to their construction and their inhibition characteristics[23-24]. The human HMGS and HMGR have been targeted successfully by drugs in the clinical treatment for high serum cholesterol levels. Statins, the inhibitors of HMGS and HMGR, are potent cholesterol lowering drugs widely used in clinical practice for primary and secondary prevention of coronary heart disease, but statins have a poor inhibition to the enzyme of gram-positive bacteria. HMG-CoA synthase catalyzes the biological Claisen condensation of acetyl-CoA with AcAc-CoA and is a member of a superfamily of acyl-condensing enzymes that include β-ketothiolases, fatty acid synthases (β-ketoacyl-acyl carrier protein synthase), and polyketide synthases[25]. Furthermore, the existence of two classes of one enzyme of the mevalonate pathway suggests that significant differences may also characterize the bacteria and human of additional enzymes of the pathway. Although the glutamate[26], cysteine[27], and histidine implicated as participating in catalysis in the avian form of the enzyme are conserved in *S. pneumoniae* HMGCoA synthase, the bacterial and animal HMG-CoA synthases exhibit only ca. 10% overall sequence identity[28]. The sequences of bacterial HMG-CoA synthases also cluster away from those of the eukaryotic synthases[21]. Although low sequence identity and remote clustering of the enzymes from different kingdoms are indicative of different HMG-CoA synthase, this inference must await confirmation by detailed structural investigations. HMGS kinetics of some species was chosen and was compared in Table 1.

TABLE 1

Kinetic Parameters of Characterized HMGS

Source	$K_m^{(\text{Acetyl -CoA})}$ (μmol/L)	*Vm* (μmol/min/mg)	References
H. brasiliensis	530±30	0.13±0.03	[29]
Avian liver cytosol	270	4.4	[30]
Blattella germanica	1500	66	[31]
E. faecalis	350	10	[15]
Brassica juncea	43	0.47	[32]
S. pneumoniae	213	4.69	This Study

Previously we have isolated and overexpressed the gene encoding HMGR from *S. pneumoniae*. The candidate antibiotics are acquired according to *S. pneumoniae* Homology modeling, and we have succeeded in screening some candidate antibiotics using the recombinant HMGR of *S. pneumoniae*. In this study, we report the expression, purification, and characterization of *S. pneumoniae* HMGS, the second enzyme in the cytoplasmic mevalonate pathway of isoprenoid biosynthesis. In addition, back mutation was conducted to alter the mutated nucleotide in the HMGS expression. The active site architecture of

avian HMGS is different from that of bacteria[7,25,28], and these differences might be exploited to develop effective inhibitors for use as antibacterial agents against pathogenic microorganism[33]. Currently, using computer to analyze three-dimensional structure and using homology modeling to search for structural analogues and then develop new drugs are a new research area. This experiment expressed the recombinant HMGS of *S. pneumoniae* successfully and obtained a reliable theoretical model of the three-dimensional dimer structure of HMGS from *S. pneumoniae*. Based on the 3D structure, virtual screening and molecular interactions between HMGS of *S. pneumoniae* and its ligands can be investigated. The biological activity of novel hits come from virtual screening could be tested by expressing the recombinant HMGS of *S. pneumoniae* presented in this study. Based on our structure, biochemical experiments can be proposed to test these models and further probe the mechanism. In addition, the structure of HMG-CoA synthase will facilitate the structure-based design of alternative drugs to cholesterol-lowering therapies or to novel antibiotics to the Gram-positive cocci, whereas the recombinant HMG-CoA synthase *S. pneumoniae* will prove useful for drug development against a different enzyme in the mevalonate pathway.

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