

## Characterization of a Functionally Active Recombinant 1-Deoxy-D-Xylulose-5-Phosphate Synthase from *Babesia bovis*

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**ABSTRACT.** The 1-deoxy-D-xylulose-5-phosphate synthase (DXS) enzyme has been characterized in other species, but not in the genus *Babesia*, which causes major losses in the livestock industries worldwide. Therefore, we isolated, cloned and expressed the wild-type *B. bovis* *dxs* cDNA in *Escherichia coli* and evaluated its enzymatic activity *in vitro*. DNA sequence analysis revealed an open reading frame of 2061 bp capable of encoding a polypeptide of 686 amino acid residues with a calculated isoelectric point of pH 6.93 and a molecular mass of 75 kDa. The expressed soluble recombinant fusion DXS protein was approximately 78 kDa, which is similar to the native enzyme identified from the parasite merozoite using anti-rDXS serum. The recombinant fusion DXS enzyme exhibited  $K_m$  values of  $380 \pm 46 \mu\text{M}$  and  $790 \pm 52 \mu\text{M}$  for D,L-glyceraldehyde 3-phosphate and pyruvate, respectively. In this work, we present the first cloning, expression and characterization of DXS enzyme from *B. bovis*.

**KEY WORDS:** *Babesia bovis*, characterize, DXS, isoprenoid biosynthesis.

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*Babesia* are tick-transmitted intra-erythrocytic protozoan parasites that infect mammals including humans and are acknowledged for their strong impact on the livestock industry and associated massive economic costs in tropical and sub-tropical areas [7, 9, 24]. *Babesia bovis* causes bovine babesiosis, which is usually characterized by extensive erythrocytic lysis leading to anemia, hemoglobinuria and even death in some cases [28]. Currently, there is no effective recombinant vaccine against apicomplexan pathogens including *Babesia* [11]. Although some compounds are available to treat babesiosis, the development of drug resistance, reinfection, side effects and residue problems are likely events. Therefore, there is a need to develop novel chemotherapeutic agents against these pathogens for the veterinary market.

The apicomplexa parasites, including *Toxoplasma*, *Plasmodium*, *Eimeria*, *Theileria* and *Babesia*, commonly contain a relic, non-photosynthetic plastid-like organelle that has been named the apicoplast and is vital for their survival [14, 22]. The recent explosion of molecular genetics tools in some of these organisms revealed that the apicoplast should greatly facilitate the identification of novel therapeutic targets [2, 26, 29]. The apicoplast harbors several metabolic pathways that supply essential biosynthetic precursors to the parasite. The synthesis of fatty acids and isoprenoid precursors is its most prominent functions, and the apicoplast has been shown to be essential for the survival of *P. falciparum*

and *T. gondii* specifically [9, 29].

The isoprenoid mevalonate independent biosynthesis of parasitic protozoa in the apicoplast is a promising chemotherapeutic target, because this pathway is different from the mevalonate pathway in mammals and is essential to such parasites. Jomaa *et al.* demonstrated that the antibiotic fosmidomycin and its derivative FR-900098, which are specific inhibitors of this pathway, are effective against malaria and babesiosis [3, 12]. The 1-deoxy-D-xylulose 5-phosphate/2-C-methyl-D-erythritol4-phosphate (DOXP/MEP) pathway of isopentenyl pyrophosphate (IPP) biosynthesis begins with the formation of DOXP through the action of the 1-deoxy-D-xylulose-5-phosphate synthase (DXS), which is the first- and rate limiting step catalyzing the formation of DOXP, by condensation of pyruvate with D-glyceraldehyde 3-phosphate (or D-glyceraldehyde) [27]. The enzyme DXS has been cloned from several higher plants [25, 31, 32], *Escherichia coli* [30], *Streptomyces* strains [13] and *Agrobacterium tumefaciens* [2], and its enzymatic kinetic parameters were determined. Some compounds or their derivatives exhibited antibacterial activity against a pathogenic bacterium by inhibiting DXS [10, 17, 19], and delete antibacterial activity was suppressed by adding 1-deoxyxylulose, a free alcohol of DOXP [18]. Consequently, DXS is an attractive target for the development of novel antibiotics, antimalarials and herbicides. The production and characterization of recombinant *Babesia* DXS would greatly enhance drug discovery efforts targeting this enzyme.

To date, there has no report on DXS from *Babesia*. Therefore, we have cloned and expressed a *B. bovis* DXS (BbDXS) cDNA in *E. coli* BL21 (DE3) and validated the activity of the recombinant BbDXS enzyme. We then used the recombinant BbDXS (rBbDXS) to generate antibodies in rabbit for characterization of a corresponding native enzyme in the parasite. The kinetic parameters of the rBbDXS

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enzyme are also reported.

## MATERIALS AND METHODS

**Parasites:** Blood infected with *B. bovis* (Shannxian), a kind gift from the Lanzhou Veterinary Research Institute, was inoculated into a splenectomized cattle immunosuppressed by dexamethasone injection. When parasitemia reached 5%, the animal was bled and the infected blood was cryopreserved in liquid nitrogen. The animal experiments in this research were approved by Gansu Provincial Science and Technology department in China and in accordance with the Animal House of Lanzhou Institute of Husbandry and Veterinary Pharmaceutical Science Instructions.

**RNA extraction and cDNA synthesis:** Total RNA was extracted from purified merozoites using the TRIZOL Reagent (Invitrogen, Carlsbad, CA, U.S.A.) and treated with RNase-free DNaseI (TaKaRa, Otsu, Japan) to remove possible contaminating DNA. Measurement of RNA concentration was conducted at OD 260 nm on a spectrophotometer (Thermo, Hudson, NH, U.S.A.). The cDNA was prepared from approximately 5 µg of the total RNA using the M-MLV First-Strand Synthesis System for qRT-PCR (Invitrogen) according to the manufacturer's instructions.

**Cloning and bioinformatic analysis of *BbDXS* gene:** Primers specific for *B. bovis dxs* were designed based the *B. bovis* T2Bo *dxs* (GenBank accession no. XM\_001611343.1). The open reading frame (ORF) of *dxs* was amplified by PCR using primers with BamHI and HindIII restriction sites (underlined), *BbDXS*-F (GGTCGGATCCATGTGTCTGATGTTACCCCTTGT) and *BbDXS*-R (GTGCAAGCTTTCATGACGCCAGGAAGTCC). The PCR conditions were as follows: initial denaturation at 94°C for 5 min, followed by 30 amplification cycles (94°C for 45 sec, 68°C for 45 sec and 72°C for 2 min) and a final extension step at 72°C for 10 min. The PCR products were purified with the DNA Purification Kit (TaKaRa) and subcloned into pGEM-T easy vector (Promega, Madison, WI, U.S.A.). The positive clone was sequenced by Sangon Biotech Co., Ltd. (Shanghai, China).

Functional domains, enzyme active sites and secondary structures of the deduced proteins were analyzed using the BLAST search tools. The characteristics of the deduced proteins were compared with those of other DXS homologs by multiple amino acid alignment analysis, and the DXS amino acid sequences of the other species were obtained from the GenBank database. Alignment of all the homologous peptides was carried out using CLUSTAL Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>) software.

**Expression and purification of *rBbDXS* protein:** The positive clones' plasmids were digested with restriction enzymes BamHI and HindIII and then inserted into a similarly digested pET-30a expression plasmid with His-tag (Clontech, Otsu, Japan). The plasmid was transformed into *E. coli* BL21 (DE3), and the transformed colony was cultured in a lysogeny broth (LB) medium containing 50 µg/ml kanamycin sodium at 37°C. When the optical density at 600 nm reached 0.8, expression of the recombinant fusion

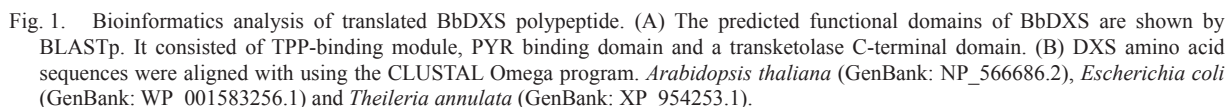
protein was induced by addition of 1 mM isopropyl β-D-thiogalactopyranoside (TaKaRa), followed by incubation for another 12 hr at 20°C. The bacteria were harvested by centrifugation and lysed by sonication in buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl, 2 mM DL-dithiothreitol, 1 mg/ml lysozyme and 0.5% Triton X-100. The recombinant fusion protein, His-rBbDXS, was purified using Ni-NTA affinity chromatography according to the manufacturer's instructions (GE Healthcare, Fairfield, CT, U.S.A.). The purified protein was analyzed on a 10% resolving gel using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The concentration of the purified rBbDXS protein was determined using the BCA protein assay (Sangon) with bovine serum albumin as the standard.

**Production of rabbit anti-rBbDXS anti-serum and identification of native *BbDXS* enzyme:** Three- to six-month-old rabbits, from which pre-immune sera were collected before immunizations, were immunized subcutaneously by injection with 2 mg purified rBbDXS protein emulsified with an equal volume of Freund's complete adjuvant (Sigma, St. Louis, MO, U.S.A.). Thereafter, three boosters consisting of 1 mg of the same antigen emulsified with Freund's incomplete adjuvant (Sigma) were administered to the rabbit via the same route at days 14, 21 and 28. Sera were collected 7 days after the last booster and checked for specific antibodies by Western blotting [23]. The animal experiments in this research were approved by Gansu Provincial Science and Technology department in China and in accordance with the Animal House of Lanzhou Institute of Husbandry and Veterinary Pharmaceutical Science Instructions.

*B. bovis* lysate was prepared as described previously [1] and separated by 10% SDS-PAGE. The native enzyme was identified by Western blotting using rabbit anti-rBbDXS serum, the lysate of health bovine erythrocytes and leukocytes as a control. The preimmune sera were reacted with the lysate as a negative control.

***rBbDXS* enzyme activity:** The rBbDXS enzyme reaction mixtures contained 120 mM Tris-HCl pH 7.5, 10 mM MgCl<sub>2</sub>, 5 mM 2-mercaptoethanol, 2 mM thiamin diphosphate, various concentrations of pyruvate-Na and D,L-glyceraldehyde 3-phosphate (D,L-GAP) or dihydroxyacetone (DHAP) and rBbDXS in a final volume of 100 µl. After incubation at 37°C, reactions were stopped by heating at 80°C for 5 min [21], and 5-µl aliquots were separated on silica gel with n-propanol/ethylacetate/H<sub>2</sub>O 5:1:3 (v/v/v) [25]. DOXP formation was monitored under 365 nm excitation after reaction with a 10% ethylenediamine sulfate solution for 5 min at 100°C [33].

For the determination of kinetic parameters, a coupled enzyme assay was used [8]. The concentrations of the substrates varied, and kinetic parameter values were calculated with GraphPad Prism v 5 Software (San Diego, CA, U.S.A.). The final enzymatic product DOXP was identified and measured by LC-MS/MS [6]. For LC-MS/MS analysis, we used a Hypersil ODS 3 µm 4.6 × 100 mm column, coupled with an Agilent 6410A triple-quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, U.S.A.) with an



## RESULTS

*Cloning and bioinformatic analysis of B. bovis DXS enzyme:* Among these enzymes involved in MEP pathway, we focused on DXS, which catalyzed the first step of the MEP pathway, making this enzyme a putative drug target. We obtained the continuous ORF of the BbDXS sequence from the cDNA. This ORF sequence (GenBank ID: KF694747) was 2,061 bp and encoded a protein of 686 amino acids with a calculated molecular mass of 75 kDa and an isoelectric point

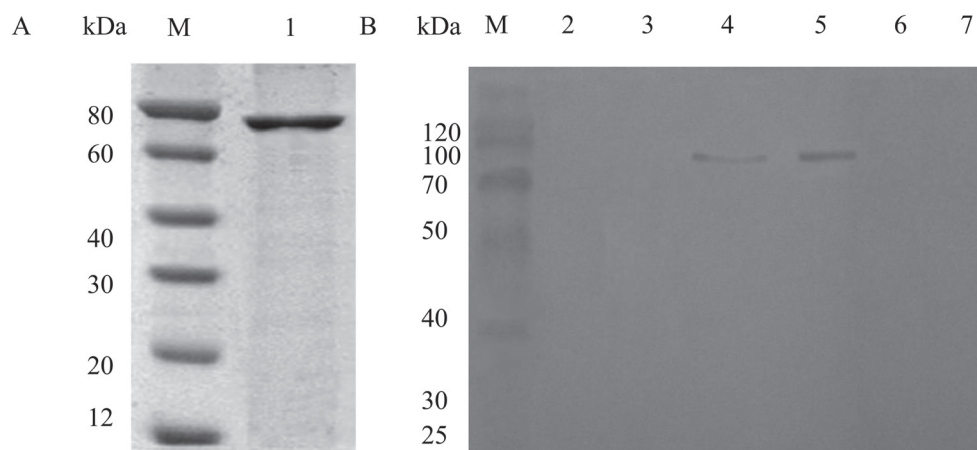


Fig. 2. Molecular characterization of native BbDXS enzyme. (A) Lane M, molecular size marker. Lane 1, purified recombinant BbDXS fused with his-tag after analysis by SDS-PAGE; (B) Lane M, molecular size marker. Lane 2 and Lane 4, Western blot analysis of the purified rBbDXS; Lane 3 and Lane 5, Western blot analysis of the lysates of *B. bovis*; Lane 6 and Lane 7, Western blot analysis of the erythrocytes and leukocyte of health bovine, respectively. The first antibody of Lane 2 and Lane 3 was preimmune sera of rabbit; the first antibody of Lane 4 to Lane 7 was rabbit anti-rBbDXS anti-serum.

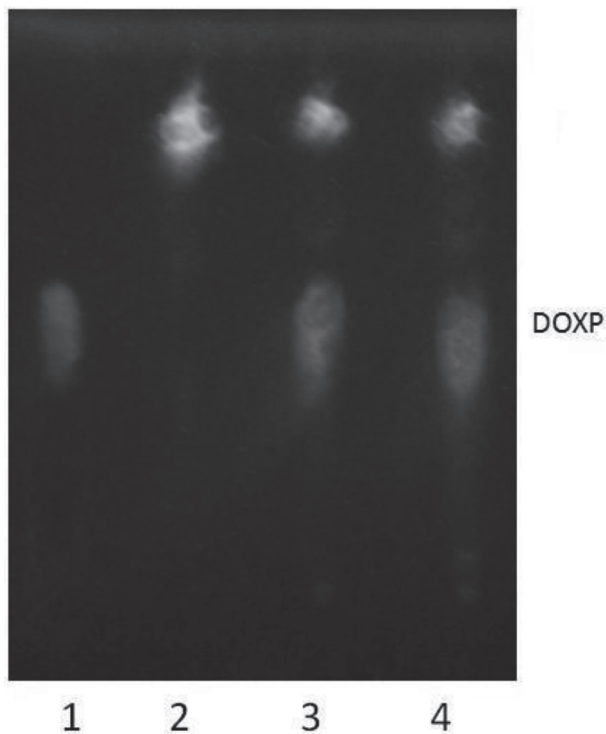


Fig. 3. Separation of the reaction mixture with different substrate by TLC. After induction of *E. coli* BL21 (DE3)/pET-30a-DXS with IPTG, purified rBbDXS was incubated with Na-pyruvate and D,L-glyceraldehyde 3-phosphate or Dihydroxyacetone (DHAP). In the product mixture separation by TLC, the product DOXP was detected under 365 nm UV either pyruvate and DL-GAP (lane3) or pyruvate and DHAP (lane4) as substrates. When TPP was omitted (lane 2), no DOXP could be detected. Lane 1 corresponds to a standard of chemically synthesized D-1-deoxyxylulose 5-phosphate (DOXP)(Sigma).

of 6.93 (GenBank ID: KF694747). Therefore, we performed BLASTp analysis of translated BbDXS polypeptide and found that it shares significant homology with the *B. bovis* T2BO DXS enzyme (GenBank ID: XM\_001611343), having 98% amino acid sequence identity (E value=0).

The predicted secondary structure of BbDXS consisted of thiamine pyrophosphate (TPP)-binding module, a pyrimidine (PYR) binding domain and a transketolase C-terminal domain (Fig. 1A), each of which bears homology to the equivalent domains in transketolase and the E1 subunit of pyruvate dehydrogenase. These predicted results indicated that the BbDXS belongs to the TPP-dependent superfamily, as the DXS enzyme is highly conserved in plants and bacteria (Fig. 1B). Residues in the active catalysis domain are highly conserved among the DXS enzymes, and weak sequence homology has also been identified with transketolase and the pyruvate dehydrogenase E1 subunit [29].

**Expression and detection of the native of BbDXS enzyme:** To complete the DXS enzyme biochemical characterization, the corresponding gene was overexpressed in *E. coli* and purified by Ni-NTA affinity chromatography. Then, antibodies were generated in rabbit for detecting the native BbDXS enzyme. Purified rBbDXS revealed a 78-kDa band on 10% SDS-PAGE, including an additional 3 kDa of the His-tag sequence (Fig. 2A). Furthermore, rabbit anti-rBbDXS enzyme serum reacted with *B. bovis* lysate to yield a specific band of approximately 75 kDa and with the purified rBbDXS to yield a 78-kDa band, but not the health bovine erythrocytes and leukocytes. However, serum of non-immunized rabbit did not react with the *Babesia* lysate (Fig. 2B).

**rBbDXS enzyme activity:** We theorized that the expressed rBbDXS is likely to be an active enzyme because BbDXS cDNA encoded an authentic protein. Therefore, we examined rBbDXS activity and found that rBbDXS could cata-



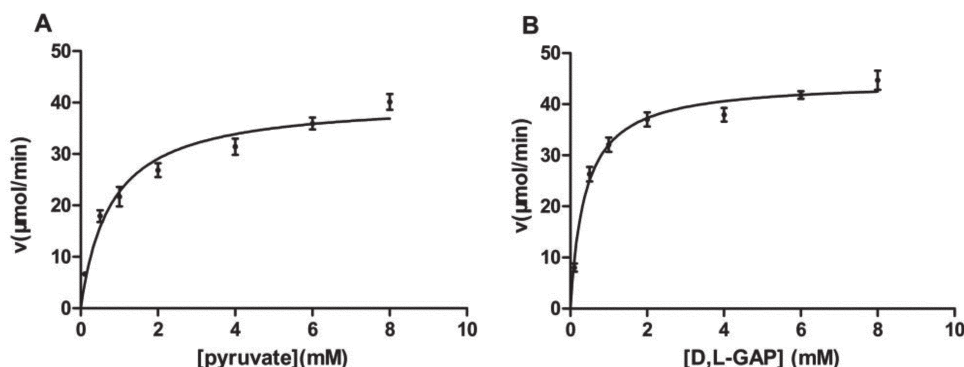


Fig. 4. Effects of substrate concentration on the activities of purified DXS for pyruvate (A) and D,L-GAP (B). DXS activities were determined in the presence of a fixed concentration of the second substrate by using a coupled assay method.

lyze the formation of DOXP using D,L-GAP or DHAP as a substrate. This demonstrates that the rBbDXS enzyme may have isomerization activity. When the reaction product was subjected to chromatography with authentic DOXP (Sigma) under the same assay conditions, no formation of DOXP was detected when TPP was omitted (Fig. 3).

The steady-state kinetic parameters for rBbDXS enzyme revealed  $K_m$  values of  $380 \pm 46 \mu\text{M}$  and  $790 \pm 52 \mu\text{M}$  for D,L-GAP and Na-pyruvate, respectively (Fig. 4). The  $K_m$  values for both pyruvate and D,L-GAP were higher than the values obtained for *Agrobacterium tumefaciens* (40.3 and 23.2  $\mu\text{M}$  for pyruvate and GAP, respectively) [15] and for *Streptomyces* sp. strain (65 and 120  $\mu\text{M}$  for pyruvate and D-GAP, respectively) [12].

## DISCUSSION

The mevalonate-independent pathway for isoprenoid biosynthesis, which generates isoprenoid precursors, is a promising chemotherapeutic target, because this pathway is different from the mevalonate pathway in mammals and very important to such pathogens [12]. The reaction, which is catalyzed by DXS enzyme in the MEP pathway, is similar to that of transketolases: a  $C_2$  unit derived from pyruvate is transferred to an aldose in a thiamine-dependent reaction [16]. Although the DXS proteins from other microorganisms and plants have been the subject of characterization and metabolic engineering studies [15], no characterization has been reported for DXS from *B. bovis*, which is one of the most important apicomplexan parasites worldwide. Therefore, we cloned and expressed the DXS-encoding genes from *B. bovis* in this study.

First, we identified the *B. bovis* *dxs* gene that encodes the putative enzyme of the MEP pathway, indicating that the pathway is present in this parasite. Multiple sequence alignments show that the deduced secondary structure of BbDXS is similar to that of other known enzymes. We found that the BbDXS polypeptide has TPP-binding module, a PYR-binding domain, and a transketolase C-terminal domain as do the already characterized DXS enzymes [30]. We also found

that amino acid residues in the catalytic domain of *Babesia* DXS and those of other species are highly conserved. These observations appear to concur with previous reports that the DXS enzyme belongs to the TPP-dependent superfamily and shares homology with the transketolases [8].

In apicomplexa, protein import into the lumen of the apicoplast is facilitated by a bipartite signaling mechanism that requires an N-terminal signal peptide followed by a transit peptide. However, application of either program to predict apicoplast targeted proteins in related apicomplexans is unreliable due to the low Adenine-Thymidine content of the genome of *Plasmodium falciparum* used to train PATS and PlasmoAP [4, 20]. Predicted signal and transit peptides for the *B. bovis* DXS protein showed that there is no signal peptide and transit peptide using SingalP, PSORT, PATS and PlasmoAP.

In this study, we successfully expressed a corresponding rBbDXS in *E. coli* and produced its anti-serum. Antisera against recombinant enzymes have been used to identify and characterize other enzymes of apicomplexan parasites, including *Babesia* species [5]. Similarly, our results revealed that rabbit antisera against rBbDXS could identify rBbDXS and native BbDXS in *B. bovis*, demonstrating that the BbDXS cDNA encoded an authentic enzyme that exists in some of the parasite stages.

Expression of the recombinant DXS enzyme enabled us to study the biochemical properties of the enzyme *in vitro*, and the determined properties of BbDXS were similar to those reported for DXS of *E. coli* and *R. capsulatus*.

In conclusion, this study reports for the first time the isolation, cloning and expression of the *B. bovis* *dxs* gene, providing a corresponding purified rBbDXS enzyme of approximately 78 kDa that is catalytically active. Our study also demonstrated the presence of an approximately 75-kDa native BbDXS in the parasite lysate by Western blotting. We showed that *B. bovis* has a catalytically active DXS enzyme and characterized the biochemical properties of rBbDXS *in vitro*. Consequently, we propose further research of the active sites of the enzyme and the study of the tertiary structure of the purified enzyme to facilitate the design of anti-*babesia*

agents that can optimally target the active sites of the enzyme.

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