*Journal of Biochemistry and Molecular Biology*, Vol. 38, No. 5, September 2005, pp. 624-631

JOURNAL OF Biochemistry and Molecular Biology

**Short communication**

# Expression, Purification and Properties of Shikimate Dehydrogenase from *Mycobacterium Tuberculosis*

**Xuelian Zhang, Shunbao Zhang, Fang Hao, Xuhui Lai, Haidong Yu, Yishu Huang and Honghai Wang***

State Key Laboratory of Genetic Engineering, Institute of Genetics, School of Life Sciences, Fudan University, Handan Road 220, Shanghai 200433, P.R. China

*Received 24 May 2005, Accepted 18 July 2005*

**Tuberculosis, caused by *Mycobacterium tuberculosis*, continues to be one of the main diseases to mankind. It is urgent to discover novel drug targets for appropriate antimicrobial agents against this human pathogen. The shikimate pathway is considered as an attractive target for the discovery of novel antibiotics for its essentiality in bacteria and absence in mammalian cells. The *Mycobacterium tuberculosis aroE*-encoded shikimate dehydrogenase was cloned, expressed and purified. Sequence alignment analysis shows that shikimate dehydrogenase of *Mycobacterium tuberculosis* exhibit the pattern of G-X-(N/S)-V-(T/S)-X-P-X-K, which is highly conserved within the shikimate dehydrogenase family. The recombinant shikimate dehydrogenase spectrum determined by CD spectroscopy showed that the percentages for α-helix, β-sheet, β-turn, and random coil were 29.2%, 9.3%, 32.7%, and 28.8%, respectively. The enzymatic characterization demonstrates that it appears to be fully active at pH from 9.0 to 12, and temperature 63°C. The apparent Michaelis constant for shikimic acid and $NADP^+$ were calculated to be about 29.5 μM and 63 μM. The recombinant shikimate dehydrogenase catalyzes the substrate in the presence of $NADP^+$ with an enzyme turnover number of 399 $s^{-1}$. Zymological studies suggest that the cloned shikimate dehydrogenase from *M. tuberculosis* has a pretty activity, and the work should help in the discovery of enzyme inhibitors and further of possible antimicrobial agents against *Mycobacterium tuberculosis*.**

**Keywords:** Enzymatic characterization, *Mycobacterium tuberculosis*, Recombinant protein, Shikimate dehydrogenase, Shikimate pathway

*To whom correspondence should be addressed.
Tel: 86-21-65643777; Fax: 86-21-65648376
E-mail: hhwang@fudan.edu.cn

## Introduction

Tuberculosis remains the leading cause of mortality due to a bacterial pathogen, and infects approximately 32% of the world's human population. The emergence of multi-drug-resistant strains of *Mycobacterium tuberculosis* and the susceptibility of patients infected with human immunodeficiency virus (HIV) to tuberculosis have fuelled the spread of the disease. Accordingly, new antimycobacterial agents are needed to treat strains of *M. tuberculosis*.

The shikimate pathway is essential for biosynthesis of aromatic amino acids and aromatic compounds in bacteria, parasites, and plants (Anton *et al*., 1988; Roberts and Coggins, 1998). The seven-step reaction in this pathway yields chorismate, which serves as a precursor for the synthesis of L-tyrosine, L-phenylalanine, L-tryptophan, and many aromatic compounds, such as foliates, ubiquinones, and napthoquinones (Roberts *et al*., 1998). The importance of shikimate pathway enzymes for metabolism in lower organisms and their absence in mammals make them attractive targets for development of antimicrobial agents and herbicides (Steinrucken and Amrhein, 1980; Davies *et al*., 1994). In mycobacteria, the shikimate pathway leads to the precursors of aromatic amino acids, ubiquinones and mycobactin. The reactions involved in the biosynthesis of shikimate should represent excellent potential target sites for chemotherapy against *M. tuberculosis*, and other mycobacterial pathogens. Thus, the mechanisms of action and properties of the enzymes involved in the biosynthesis of shikimate represent important information for the design and development of new antimycobacterial drugs.

Homologs to enzymes in the shikimate pathway have been identified in the complete genome sequence of *M. tuberculosis* H37Rv strain (Cole *et al*., 1998). *M. tuberculosis aroE*-encoded (Rv2552c) shikimate dehydrogenase (SD; EC 1.1.1.25) catalyzes the fourth step of the shikimate biosynthetic pathway and is responsible for the reduction of 3-dehydroshikimate to shikimate in the presence of NADPH (Parish and Stoker, 2002). Magalhães and coworkers reported

the functional recombinant SD could be expressed in *Escherichia coli* (*E. coli*) BL21 (DE3)(Magalhães *et al.*, 2002). However, some enzymatic properties of recombinant SD from *M. tuberculosis* were unknown. In this report, we describe the expression and purification of the *M. tuberculosis aroE* gene in *Escherichia coli* system. The biochemical and potent enzymatic properties of recombinant have been determined. The purification and properties of wild-type shikimate dehydrogenase from mycobacteria have not been reported. We thought this work presented here should be appropriate to know the nature of the *M. tuberculosis* protein.

## Materials and Methods

**Materials** All the chemicals were purchased from Sigma Chemical Co except when otherwise noted. Ni-NTA agarose was obtained from Qiagen, and used according to the manufacturer's recommendations. LB broth was from Merck Co. All DNA manipulation enzymes, including restriction endonucleases, ligase and pfu DNA polymerase, were supplied by New England Biolabs.

**Cloning of *M. tuberculosis aroE* gene** A 810 bp *aroE* gene was amplified from *M. tuberculosis* genomic H37Rv DNA by PCR using oligonucleotide primers 2552cFP 5-GGGCATATGAGCGA AGGTCCCAAAAAAGCCG-3 and 2552cRP 5-ACAAAGCTTCT AGTCCAACGCGGCCAGCGCG-3. The *Nde*I site and *Hin*d site were introduced to the forward and the reverse primer, respectively. The PCR amplification was done at 10% DMSO (Magalhães *et al.*, 2002) by using the pfu DNA polymerase with a 65°C annealing temperature. The PCR product was ligated to the pET28b (Novagen, Madison, USA) vector to generate the recombinant plasmid pET28b-2552c. The recombinant plasmid was transformed in the *E. coli* strain BL21 (DE3) (Novagen) cultured with the kanamycin.

**Sequence analysis** Open reading frames Rv2552c was identified by BLAST alignment with predicted amino acid sequences on GenBank (http://www.ncbi.nih.gov). Multiple amino acid alignments were performed CLUSTALX program (Thompson *et al.*, 1997).

**Expression of recombinant SD** The *E. coli* BL21 (DE3) cells with recombinant plasmid was cultured in L-broth containing 50 μg $mL^{-1}$ kanamycin. Cells were incubated at 220 rpm at 37°C. Moreover, the cells were grown to an optical density of 0.6. At that point, 0.5 mM IPTG was added and the cells were incubated for an additional 5 h.

**Purification of recombinant SD** Host cells expressing recombinant SD protein were harvested by centrifugation, and subjected to freeze/thaw at −80°C and ice/water bath (Magalhães *et al.*, 2002), then suspended in buffer A (50 mM $NaH_2PO_4$, 300 mM NaCl, pH 8.3, 0.1% Triton X-100) and sonicated on ice. The mixture was centrifuged to yield a clear supernatant, which was loaded onto a column with Ni-NTA resin pre-equilibrated in buffer A and mixed gently for 1 h at 4°C. The column was washed with buffer B (50 mM $NaH_2PO_4$, 300 mM NaCl, pH 8.3 and 20 mM imidazole), buffer C (50 mM $NaH_2PO_4$, 300 mM NaCl, pH 8.3 and 30 mM imidazole) and buffer D (50 mM $NaH_2PO_4$, 300 mM NaCl, pH 8.3 and 40 mM imidazole) for several times and eluted with buffer F (50 mM $NaH_2PO_4$, 300 mM NaCl, pH 8.3 and 150 mM imidazole), then purified protein was pooled and dialyzed against buffer G (5 mM Tris-HCl, pH 8.0) to remove imidazole. The appropriate concentration of SD was thus concentrated by ultrafiltration with Amicon centrifugal filter device. All purification, dialysis and concentration procedures were performed at 4°C. Protein concentrations were determined by Bradford assay using bovine serum albumin (BSA) as the standard. Predicted size expression of the $(His)_6$-*aroE* polypeptide (29 kDa) was confirmed by SDS-PAGE. Purified protein was analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) using Voyager-DE-PRO instrument (Applied Biosystems, Foster City, USA).

**Enzymatic activity assay** SD catalyzes the NADPH-dependent reduction of 3-dehydroshikimate to form shikimate and $NADP^{+}$. Enzyme activity was assayed by continuously monitoring the increase in NADPH absorbance at 340 nm ($\varepsilon NADPH = 6.22 \times 10^3$ $M^{-1}$ $cm^{-1}$) (Chaudhuri, *et al.*, 1987). All spectrophotometric assays were performed at 25°C, and the increase in NADPH was monitored at 340 nm. The assays were conducted in a final volume of 100 μL, containing the following components: 100 mM Tris-HCl, pH 9.0, 4 mM shikimic acid, 2 mM $NADP^+$ and an appropriate amount of enzyme. All of the components except for the SD enzyme were premixed in a reservoir and dispensed. The reaction mixture was incubated at 25°C for 5 min to reach a stable background. The SD enzyme was added in the end to trigger the reaction.

Michaelis constants were determined for the two substrates, shikimic acid and $NADP^+$ in the forward reaction. The Km estimated at fixed another substrate concentration is not the true values but rather apparent values. Therefore these parameters are referred as apparent Km. The recombinant SD activity was measured at various concentrations (0.02-5 mM) of Shikimic acid with 2 mM $NADP^+$ at pH 9.0 and 25°C. Concentration of the $NADP^+$ for determination of apparent Km varied between 0.025-4 mM, and Shikimic acid concentration was 2 mM at 25°C at pH 9.0.

**Properties and stability of the recombinant SD** To determine the optimum pH of the recombinant SD, buffers with different pH were used in the assay mixture. For pH profile analysis, the activity of SD was measured in different pH buffers (100 mM Tris-HCl for pH 6.0-9.5, and 3-(cyclohexylamino) propane sulfonic acid-NaOH for pH 10-13). As far as the effect of temperature on recombinant SD is concerned, the enzymatic activity assays were processed from 10-90°C. The heat stability of recombinant SD was measured by preincubated at temperatures 50, 60 and 70°C ranging from 0 min to 10 min. All results shown were the average of three different experiments.

**Circular dichroism (CD) measurement of recombinant SD** To determine secondary structure elements of the recombinant SD, far-UV CD spectra were recorded at 25°C with a Jasco-715 spectrophotometer (Jasco, Tokyo, Japan) equipped with RTE bath/circulator (NESLAB RTE-111; NESLAB, Tokyo, Japan). Protein concentration of 0.132 mg $ml^{-1}$ in 50 mM Tris-Cl, pH 7.8, was used. The spectra were recorded from 190 nm to 300 nm with a

resolution of 0.2 nm and accumulated for five scans. Secondary structure parameters were estimated by the computer program PROSEC derived by Yang and coworkers (Yang *et al.*, 1986).

## Results and Discussion

**Cloning, expression and purification of the recombinant SD** A 810 bp PCR product was amplified from *M. tuberculosis* H37Rv genomic DNA and inserted into the expression vector pET28b. The gene was sequenced to confirm the fidelity of the amplification.

Expression of SD using pET28b showed presence of higher amounts of recombinant protein as inclusion bodies than soluble fraction in supernatant. There is a rare codon (CUA) for leucine at position 254 in *M. tuberculosis aroE* gene, insufficient tRNA could result in amino acid misincorporation that might lead to express nonproperly folded recombinant protein (Kurland and Gallant, 1996). There have been many attempts to refold the protein denatured in 8M urea using different refolding buffer, pH, temperature, and denature protein concentrations. Disappointingly, the attempts to yield large amounts of biologically active product were unsuccessful. A number of trials showed that the soluble protein quantities could be improved by repeated freezing /thawing and then sonication. Meanwhile, the cells were allowed to remain on ice for 15 min before being subjected to sonication.

The recombinant SD having a $(His)_6$ tag could be purified on a nickel column as has been widely used for various recombinant protein. However, soluble recombinant SD has very low binding ability to Ni-NTA resin during purification. In order to increase the binding ability to Ni-NTA resin, the crude extract was applied to a nickel ion column and mix gently for 1 h at 4°C. On the other hand, the pH value and composition of all buffers were checked and chosen the conditions including 50 mM $NaH_2PO_4$, pH 8.3 and 300 mM NaCl, in which purified soluble recombinant protein was obtained. As shown in Fig. 1, a single band with a molecular mass about 29 kDa was detected in the elution fraction.

The overall yield of the purification was 57.5% and the purified protein was estimated to be about 0.68 mg from 1 L culture medium (Table 1). Although obtained recombinant protein from 1 L culture medium was a little lower than those recombinant proteins expressed high in the soluble form, the recombinant SD was a pretty active and functional enzyme. We observed that recombinant SD catalyzes the shikimate acid at 2 mM in the presence of 2 mM $NADP^+$ with an enzyme turnover number of 399 $s^{-1}$. The catalytic rate for shikimate was comparable with the published rate for wild-type *E. coli* AroE (430 $s^{-1}$) (Table 2) (Chaudhuri *et al.*, 1987), In contrast, eukaryotic SDs exhibited lower activities ranging from 2.25 to 160 U/mg (Dowsett and Corbett, 1971; Barea *et al.*, 1978; Polley, 1978; Lourenco *et al.*, 1991).

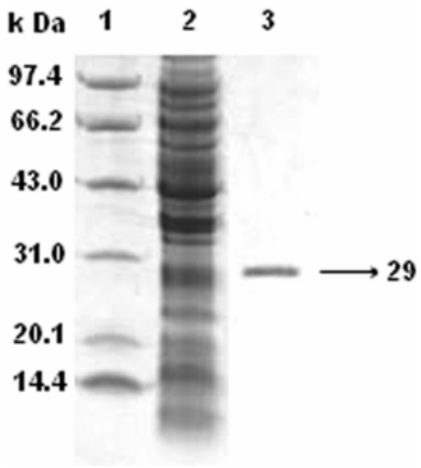


**Fig. 1.** Purification of recombinant shikimate dehydrogenase on the nickel column. Lane 1, protein standards; lane 2, crude *Escherichia coli* supernatant fraction before application to column; lane 3, fraction eluting with 150 mM imidazole. Proteins were visualized with Coomassie blue stain.

The mass of purified recombinant protein was conformed by MALDI-TOF (Fig. 2), which was in accordance with the theoretical mass prediction of protein. The measured mass for recombinant protein was 29,314.2754 ± 30 Da (the predicted mass is 29,340.50 Da).

**Sequence analysis of *M. tuberculosis* SD** BLAST analysis of the predicted amino acid sequence of Rv2552c shows homology with a number of proposed shikimate dehydrogense, e.g., *E. coli* SD (GI:16131162, 24% identity), *M. tuberculosis* CDC1551 (GI:15842090, 86% identity), *Mycobacterium bovis* AF2122/97 (GI:31793735, 85% identity), and *Mycobacterium leprae TN* (GI:15827177, 70% identity). Fig. 3 shows the result of the deduced amino acid sequence comparison of SD

**Table 1.** Purification of the recombinant SD

| Steps | Total protein (mg) | Total activity (U/mg) | Specific activity (U/mg)[a] | Yield (%) |
|---|---|---|---|---|
| Supernatant proteins[b] | 315 | 976.5 | 3.1 | 100 |
| Ni-chelation column | 0.8 | 610.4 | 763 | 62.5 |
| Concentration[c] | 0.68 | 561.7 | 826 | 57.5 |

[a]The enzyme activities were determined at pH 9.0 and 25°C using 2 mM shikimic acid and 2 mM $NADP^+$ as substrates. One unit of enzyme activity (U) is defined as the amount of enzyme catalyzing the conversion of 1 μmol substrate per minute.
[b]The supernatant proteins was from 1 L culture medium.
[c]The purified protein was concentrated by ultrafiltration with Amicon centrifugal filter device.

**Table 2.** Activity and kinetic parameters of purified SD enzymes from recombinant and wild-type sources

| Sources | Specific activity (U/mg) | Kcat ($s^{-1}$) | Km (shikimic acid) (mM) | Km ($NADP^+$) (mM) | References |
|---|---|---|---|---|---|
| Recombinant SD from *M. tuberculosis* | 826 | 399 | 0.030 | 0.063 | This work |
| *Xanthomons oryzae* | 5.4 | 0.0028 | 0.050 | No[b] | (GOEL, *et. al*., 2001) |
| *E. coli* | 860 | 430 | 0.055 | 0.031 | (Chaudhuri, *et. al*., 1987) |
| Recombinant SD from *E.coli* | 444 | 237 | 0.065 | 0.056 | (Michel, *et al*., 2003) |
| *Neurospora crassa* | 86.87 | 59 | 1.18 | No[b] | (Barea, *et al*., 1978) |
| Pea epicotyls (*pisum satium*) | 2.25 | -[a] | 0.34 | 0.070 | (Dowsett, *et al*., 1972) |
| Pea seedling (*pisum satium*) | 0.279 | -[a] | 0.19-0.28 | 0.007 | (Balinsky, *et al*., 1971) |

[a]The molecular weight is unknown and enzyme turnover number cannot be provided.
[b]No provided in reference.

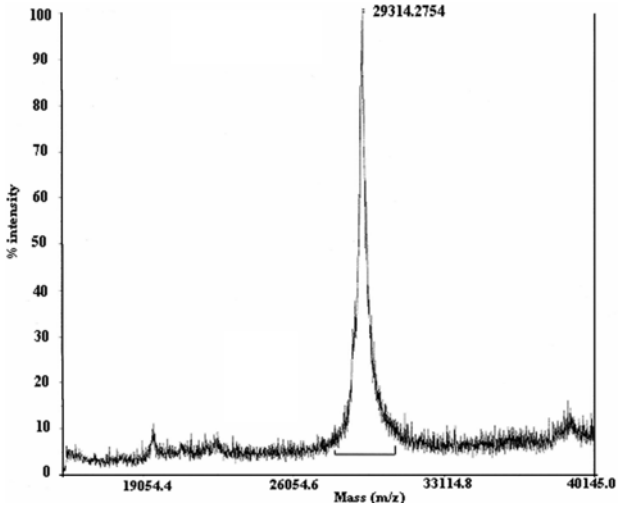


**Fig. 2.** MALDI-TOF of mass spectrum of recombinant protein. The spectrum shows the mass of recombinant protein was 29,314.2754 ± 30 Da.

in H37Rv strain with amino acid sequence of SD from several other model organisms. Alternating residues from Gly61-Lys69 exhibit the following pattern of conservation G-X-(N/S)-V-(T/S)-X-P-X-K, (where X represents any amino acid), Which create a conserved sequence in shikimate dehydrogenase. Crystallization and preliminary analysis of *Methanococcus jannaschii* SD explained the conserved pattern creating a surface patch that leads to the active site cleft (Padyana and Burley, 2003). Asn90 and Thr91 are conserved in SD sequences. Asp105, Thr104, Gln247 and Thr319 which are also highly conserved within the SD family are seen at the C terminus. It is likely that the Lys69, Asn90, Asp105 and Glu259 represent residues involved in catalytic reduction of 3-dehydroshikimate to shikimate (Padyana and Burley, 2003). This homology analysis provides a wealth of information, which has not only helped to clarify the possible function of unannotated sequences, but also can serve as the basis for future functional studies and prove crucial in the design of new antimicrobial.

**Properties of the recombinant SD** In the further experiments, we have investigated the effects of pH and temperature on recombinant SD. The activity of the purified recombinant SD showed a linear increase with increasing protein concentration, from 1-20 μg of protein per incubation. In order to investigate the apparent Km of recombinant SD, Shikimic acid, concentrations from 0.02 to 5 mM, and $NADP^+$ from 0.025 to 4 mM were used and activity was monitored. We observed that at higher shikimic acid concentrations above 2.4 mM the enzymatic reaction was found to be inhibitory (Fig. 4A, inset A). The kinetic parameter Km about 29.5 μM was determined in the presence of varied (0.02-2.4 mM) concentration of shikimic acid (Fig. 4A), and the Km value for $NADP^+$ was 63 μM (Fig. 4B) at pH 9.0, at 25°C.

We compared the Michaelis constants with those of wild-type shikimate dehydrogenase isolated from natural sources (Table 2). Although the Km for substrates of recombinant SD was not as same as those of wild-type origins, recombinant SD and AroEs from bacteria display a fairly equivalent affinity for their substrates, as shown by the similar range of their Km values. In contrast, the enzymatic Km values for shikimic acid were quite high, and AroEs from plants had a low affinity for the shikimic acid compared with those from bacteria. Earlier study reported that the Michaelis constants were affected by pH (Balinsky *et al*., 1971). The Km values for shikimic acid were of the same order of magnitude as the apparent Michaelis constants of 0.09 mM for mung bean seedlings at pH 8 and 0.43 mM for tea shoots at pH 9 (Balinsky *et al*., 1971).

As shown in Fig. 5, the enzyme was almost no activity at acid pH (6.0). The pH curve indicated that SD had higher activity at pH from 9.0 to 12. The result was similar to that of *E. coli*, and its higher activity was valued at pH 10.5 (Chaudhuri and Coggins, 1985). In addition, the enzymatic activity of SD gradually increased between 20 and 63°C and decreased from 63 to 90°C (data not shown). The temperature stability profiles of SD were also investigated. Fig. 6 indicated that the SD lost almost all of its activity in 1 min at 70°C. At 50°C, the enzymatic activity retained 63% after 10 min of incubation at 50°C. Resent study reported a thermostable

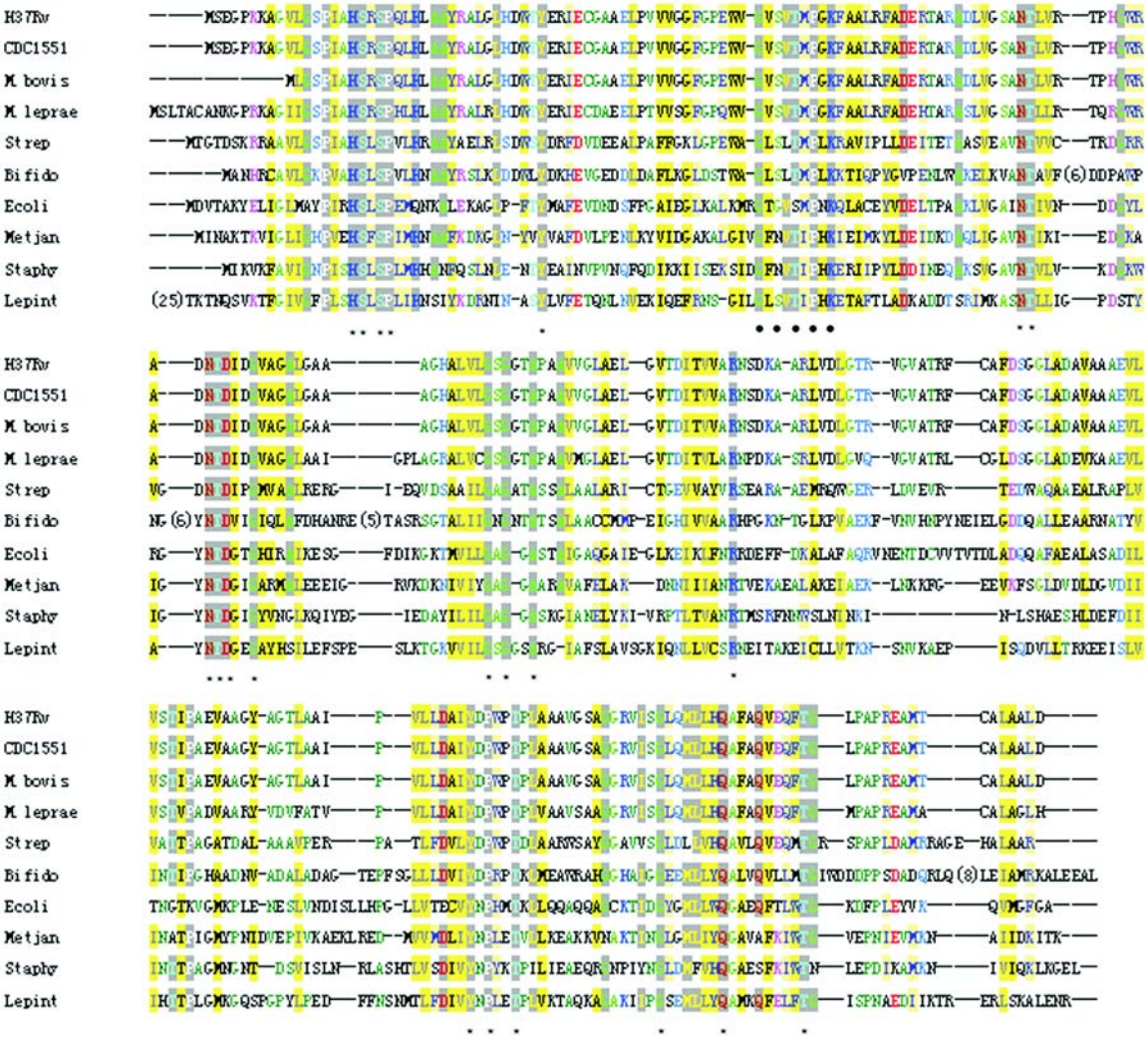


**Fig. 3.** Alignment of *Mycobacterium tuberculosis aroE* predicted amino acid sequence with those of homologous genes from other organisms. The sequences were aligned by ClustalX and followed minimal manual editing. The alignment was colored and the 80% consensus sequence calculated using Chroma. Amino acids that are identical in all ten organisms are indicated by ★; ● indicates the conservative pattern of active site. The sequences are denoted by the species abbreviation. The species abbreviations are: H37Rv, *Mycobacterium tuberculosis* H37Rv (GI: 15609689); CDC1551, *Mycobacterium tuberculosis* CDC1551 (GI: 15842090); M. bovis, *Mycobacterium bovis* AF2122/97 (GI:31793735); M.leprae, *Mycobacterium leprae* (GI:13092725); Strep, *Streptomyces avermitilis* MA-4680 (GI:29833395); Bifido, *Bifidobacterium longum* (GI:23465284); E coli, *Escherichia coli* K12 (GI:16131162); Metjan, *Methanocaldococcus jannaschii* DSM2661 (GI:15669272); Staphy, *Staphylococcus epidermidis* ATCC 12228 (GI:27468200); Lepint, *Leptospira interrogans serovar* Lai str.56601 (GI:24213338).

shikimate 5-dehydrogenase from archaeon, and the enzyme owned a half-life activity at 87°C for 2 hours (Lim *et al*., 2004). Thermal stability and activity of the enzyme were greatly enhanced by addition of NaCl or KCl (Barea *et al*., 1978; Lim *et al*., 2004).

In experiment, we found that the purified recombinant SD was stable to freezing and maintained its activity for at least two weeks when stored at 4°C and for several months at −70°C. Earlier workers reported the wild-type enzyme from *pisum sativum* was stable to be kept for at least 3 months at −15°C with little activity loss (Balinsky *et al*., 1971) and purified *E.coli* SD was stable for 6 months stored −20°C in buffer containing 50% (v/v) glycerol and 1 mM benzamidine (Chaudhuri *et al*., 1987). In addition, the enzyme was also quite stable to some detergents when it was incubated in various detergents at different concentrations. The detergents such as Nonidet P-40, and Triton X-100 were relatively innocuous and even at 0.1-0.5% the relative activity was significantly increased from 120% to 190%. We used 0.1% Triton X-100 to lyse cell, Triton X-100 was not in the buffer used to elute the SD from the nickel ion columns. The enzymatic activity was rapidly lost in the presence of increasing concentrations of SDS, and at 0.02% of these detergents there was almost complete loss of activity.

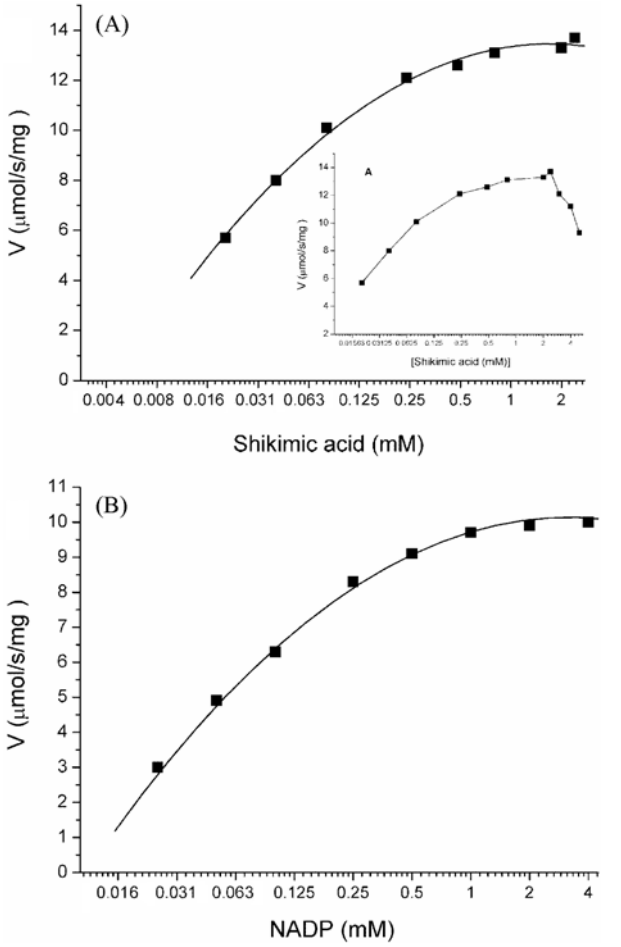


**Fig. 4.** Kinetic profiles for the determination of recombinant shikimate dehydrogenase kinetic parameters. A, increasing concentration of shikimic acid with $NADP^+$ at 2 mM. Inset A, the effect of excess shikimate dehydrogease concentration (up to 5 mM) on enzymatic rate. B, increasing $NADP^+$ concentration of with shikimic acid at 2 mM.

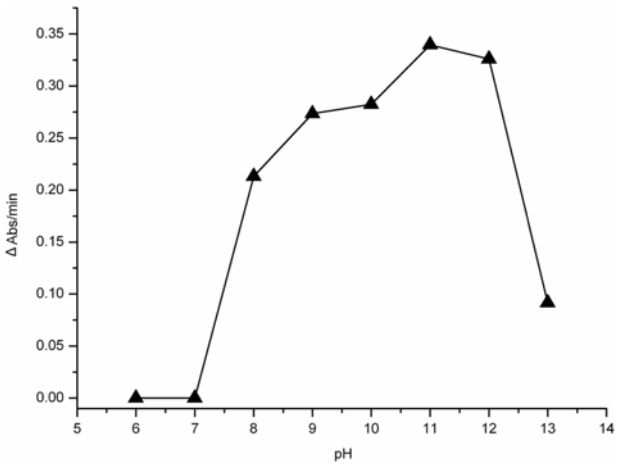


**Fig. 5.** Effect of pH of incubation mixture on activity of recombinant shikimate dehydrogenase. Assay mixtures were prepared and detected as described in "Materials and methods" but contained buffers at various pH values (6-13) as shown in the figure.

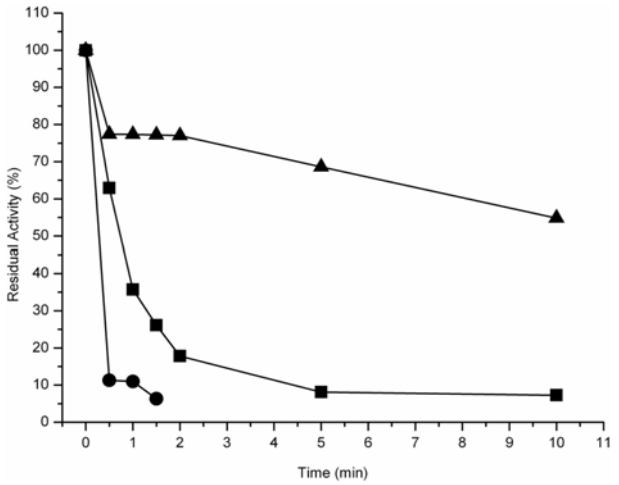


**Fig. 6.** The heat stability of recombinant shikimate dehydrogenase. Recombinant protein which was incubated for various times at 50°C (▲), 60°C (■) and 70°C (●) was added into assay mixtures at the times indicated in the figure to trigger the reaction. Enzyme activity was described as residual activity and the activity of recombinant SD without incubation was set to 100%.

The SD spectrum showed that the percentages for α-helix, β-sheet, β-turn, and random coil were 29.2, 9.3, 32.7%, and 28.8%, respectively. Recently, the crystal structures of AroE from *E. coli, Methanococcus jannaschii*, and *H. influenza*e and of YdiB from *E.coli* and *H. influenzae* were solved (Maclean *et al.*, 2000; Benach *et al.*, 2003; Padyana and Burley, 2003; Ye *et al.*, 2003; Singh *et al.*, 2005). All of these structures revealed a common fold comprising two α/β domains. We compared the secondary structure of recombinant SD obtained by CD spectroscopy with those calculated from reported crystallographic structures. The percentages for α-helix and random coil of the recombinant SD were similar to 33% and 32% calculated from the crystallographic structures, respectively. The percentages for β-sheet of cloned protein (9.3%) was lower than that reported for its homologues (Benach *et al.*, 2003; Padyana and Burley, 2003; Ye *et al.*, 2003; Singh *et al.*, 2005). The β-sheets in proteins are often bent and/or twisted and show large variation of the (Φ, ψ) angles than that in α-helices (Sreerama and Woody, 2000), and all of the methods to analyze protein secondary structure from CD spectra did good job of predicting α-helix, but they varied greatly in their ability to estimate β-sheet and turn (Greenfield, 1996). Thus, it is unsurprising that recombinant protein secondary structure obtained by CD spectroscopy does not completely correspond to known structural composition.

Polyclonal antibody against the purified recombinant SD protein from *M. tuberculosis* H37Rv reacted with crude extract of two other mycobacteria strains (Bacille Calmette-Guerin (BCG) and *M. smegmatis*) at about 28 kDa (data not

shown). Thus, antibody made with the SD protein from *M. tuberculosis* H37Rv has cross reaction with the protein in *BCG* and *M. smegmatis*.

In this report, we describe the amino acid sequence alignment, purification and the enzymatic properties of shikimate dehydrogenase from *M. tuberculosis*. Sequence alignment analysis of SD shows that *M. tuberculosis* exhibits the highly conserved G-X-(N/S)-V-(T/S)-X-P-X-K pattern of the SD family and mycobacteria share highly similar. Almost 68% of the amino acids in the *M. tuberculosis* SD are hydrophobic (*i.e.*, 52 Ala, 32 Gly, 28 Leu, 15 Pro, 27 Val, 4 Lys, 4 Met, 7 Phe, and 4 Trp). The hydrophobic nature of the protein may account for some of its unusual properties. For example, when *E. coli* cells transfected with *aroE* gene were broken by freezing /thawing and sonication, it is shown that presence of higher amounts of recombinant protein as inclusion bodies than soluble fraction in supernatant.

It is prospective that the shikimate dehydrogenase should represent excellent potential target sites for chemotherapy against *M. tuberculosis*, and other mycobacterial pathogens, for shikimate is an essential molecule for biosynthesis of aromatic amino acids. This present work should be appropriate to know the nature of the *M. tuberculosis* protein and to supply a potential platform to discover inhibitors of SD and possible antimicrobial agents against *M. tuberculosis*. We are designing experiments to screen high potent and effective anti-tuberculosis drugs based on our experimental results described in this paper.

**Acknowledgments** This research was supported by the State Basic Research Development Program of China (973 Program, No. 2002CB512804) and Shanghai Postdoctoral Science Foundation of China (No. 04R214132).

## References

Anton, I. A. and Coggins, J. R. (1988) Sequencing and overexpression of the *Escherichia coli* aroE gene encoding shikimate dehydrogenase. *Biochem. J.* **249**, 319-326.

Balinsky D., Dennis A. W. and Cleland W. W. (1971) Kinetic and isotope-exchange studies on shikimamte dehydrogense from *pisum sativum*. *Biochem* **10**, 1947-1952.

Barea, J. L. and Giles, N. H. (1978) Purification and characterization of quinate (shikimate) dehydrogenase, an enzyme in the inducible quinic acid catabolic pathway of *Neurospora crassa*. *Biochim. Biophys. Acta.* **524**, 1-14.

Benach, J., Lee, I., Edstrom, W., Kuzin, A. P., Chiang, Y., Acton, T. B., Montelione, G. T. and Hunt, J. F. (2003) The 2.3-Å Crystal structure of the shikimate 5-dehydrogenase orthologue YdiB from *Escherichia coli* suggests a novel catalytic environment for an NAD-dependent dehydrogenase. *J. Bio. Chem.* **278**, 19176-19182.

Chaudhuri, S. and Coggins J. R. (1985) The purification of shikimate dehydrogenase from *Escherichia coli*. *Biochem. J.* **226**, 217-223.

Chaudhuri, S., Anton, I. A. and Coggins, J. R. (1987) Shikimate dehydrogenase from *Escherichia coli*. *Methods Enzymol.* **142**, 315-320.

Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S., Barry III, C. E., Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, S., Osborne, J., Quail, M. A., Rajandream, M. A., Rogers, J., Rutter, S., Seeger, K., Skelton, S., Squares, S., Sqares, R., Sulston, J. E., Taylor, K., Whitehead, S. and Barrell, B. G. (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**, 537-544.

Davies, G. M., Barrett-Bee, K. J., Jude, D. A., Lehan, M., Nichols, W. W., Pinder, P. E., Thain, J. L., Watkins, W. J. and Wilson, R. G. (1994) (6S)-6-fluoroshikimic acid, an antibacterial agent acting on the aromatic biosynthetic pathway. *Antimicrob. Agents Chemother.* **38**, 403-406.

Dowsett, J. R. and Corbett, J. R. (1971) The purification and properties of shikimate dehydrogenase. *Biochem. J.* **123**, 23.

Goel A. K, Rajagopal L. and Sonti, R. V. (2001) Pigment and virulence deficiencies associated with mutations in the aroE gene of Xanthomonas oryzae pv. *Oryzae. Appl. Environ. Microbio.* **67**, 245-250.

Greenfield, N. J. (1996) Methods to estimate the conformation of proteins and polypeptides from circular dichroism. *Data Anal. Biochem.* **235**, 1-10.

Knaggs, A. R. (2003) The biosynthesis of shikimate metabolites. *Nat. Prod. Rep.* **20**, 119-136.

Kurland, C. and Gallant, J. (1996) Errors of heterologous protein expression. *Curr. Opin. Biotechnol.* **7**, 489-493.

Lim, S., Schroder, I. and Monbouquette, H. G. (2004) A thermostable shikimate 5-dehydrogenase from the archaeon *Archaeoglobus fulgidus*. *FEMS Microbiol Lett.* **238**, 101-106.

Lourenco, E. J., Silva, G. M. L. and Neves, V. A. (1991) Purification and properties of shikimate dehydrogenase from cucumber (*Cucumis sativus L.*). *J. Agric. Food Chem.* **39**, 458-462.

Maclean, J., Campbell, S. A., Pollock, K., Chackrewarthy, S., Coggins, J. R. and Lapthorn, A. J. (2000) Crystallization and preliminary X-ray analysis of shikimate dehydrogenase from *Escherichia coli*. *Acta Crystallogr. Sect. D.* **56**, 512-515.

Magalhães, M. L. B., Pereira, C. P., Basso, L. A. and Santos, D. S. (2002) Cloning and expression of functional shikimate dehydrogenase (EC 1.1.1.25) from Mycobacterium tuberculosis H37Rv. *Protein Expression Purification* **26**, 2659-2664.

Michel, G., Roszak, A. W., Sauve, V., Maclean, J., Matte, A., Coggins, J. R., Cygler, M. and Lapthorn, A. J. (2003) Structures of shikimate dehydrogenase AroE and its paralog YdiB. *J. Biol. Chem.* **278**, 19463-19472.

Padyana, A. K. and Burley, S. K. (2003) Crystal structure of shikimate 5-dehydrogenase (SDH) bound to NADP: insights into function and evolution. *Structure* **11**, 1005-1013.

Parish, T. and Stoker, N. G. (2002) The common aromatic amino acid biosynthesis pathway is essential in Mycobacterium tuberculosis. *Microbiology* **148**, 3069-3077.

Polley, L. D. (1978) Purification and characterization of 3-dehydroquinate hydrolase and shikimate oxidoreductase. *Biochim.Biophys. Acta.* **526**, 259-266.

Roberts, F., Roberts, C. W., Johnson, J. J., Kyle, D. E., Krell, T.,

Coggins, J. R., Coombs, G. H., Milhous, W. K., Tzipori, S., Ferguson, D. J. P., Chakrabarti, D. and Mcleod, R. (1998) Evidence for the shikimate pathway in apicomplexan parasites. *Nature* **393**, 801-805.

Singh, S., Korolev, S., Koroleva, O., Zarembinski, T., Collart, F., Joachimiak, A. and Christendat, D. (2005) Crystal Structure of a novel shikimate dehydrogenase from *Haemophilus influenzae*. *J. Bio. Chem*. **280**, 17101-17108.

Sreerama, N. and Woody, R. W. (2000) Estimation of protein secondary structure from circular dichroism spectra: comparison of CONTIN, SELCON, and CDSSTR methods with an expanded reference set *Anal. Biochem*. **287**, 252-260.

Steinrucken, H. C. and Amrhein, N. (1980) The herbicide glyphosate is a potent inhibitor of 5-enolpyruvyl-shikimic acid-3-phosphate synthase. *Biochem. Biophys. Res. Commun*. **94**, 1207-1212.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic. Acids Res*. **24**, 4876-4882.

Yang, J. T., Wu, C. S. and Martinez, H. M. (1986) Calculation of protein conformation from circular dichroism. *Methods Enzymol*. **130**, 208-269.

Ye, S., Delft, F., Brooun, A., Knuth, M. W., Swanson, R. V. and McRee D. E. (2003) The crystal structure of shikimate dehydrogenase (AroE) reveals a unique NADPH binding mode. *J. Bacteriol*. **185**, 4144-4151.