Identification and characterization of serine acetyltransferase encoded by the *Mycobacterium tuberculosis* Rv2335 gene

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Abstract. Serine acetyltransferase (CysE) is the first enzyme involved in the two-step enzymatic pathway of L-cysteine biosynthesis in bacteria and plants, but not in humans. CysE catalyzes the biosynthesis of *O*-acetyl-L-serine and CoA from L-serine (L-Ser) and acetyl-CoA (AcCoA). *Mycobacterium tuberculosis* (*M. tuberculosis*) Rv2335 was predicted as the *cysE* gene encoding serine acetyltransferase. In this study, the *M. tuberculosis* Rv2335 gene was cloned and the CysE protein was expressed in *E. coli* BL21 (DE3). The *M. tuberculosis* CysE protein was purified by Ni^{2+} affinity chromatography and confirmed by SDS-PAGE, western blotting and mass spectrometry. The serine acetyltransferase activity of the *M. tuberculosis* CysE protein was detected using Ellman's reagent. *M. tuberculosis* CysE displayed optimal activity at pH 7.5 and 37˚C. The Michaelis constant for AcCoA and L-Ser was 0.0513±0.0050 and 0.0264±0.0006 mM, respectively. The maximum velocity (V_{max}) for CysE was 0.0073±0.0005 mM/min. The CysE assay and the determination of the kinetic parameters of *M. tuberculosis* CysE may be helpful for screening its inhibitors in anti-tuberculosis drug discovery.

Introduction

Although tuberculosis (TB) is an ancient disease resulting from infection with *Mycobacterium tuberculosis* (*M. tuberculosis*), it remains a great threat to both individual and public health throughout the world. It is reported that approximately one-third of the world's population has been latently infected (1). The prevalence of human immunodeficiency virus has enhanced the spread of multi-drug resistant and extensively drug resistant tuberculosis strains, and the morbidity and mortality of TB have been rising yearly without much curative success using existing anti-TB drugs (2-4). Therefore, it is a matter of urgency to discover targets for new anti-TB drugs. Serine acetyltransferase (CysE) is involved in the biosynthesis of cysteine, which catalyzes the conversion of acetyl-CoA (AcCoA) and L-serine (L-Ser) to CoA and *O*-acetyl-L-serine (OAS) (5,6). This reaction is the first step in the two-step biosynthesis of L-cysteine in microorganisms and plants (7,8). Because of the differing pathways for cysteine anabolism in humans and microorganisms (9), serine acetyltransferase exists only in microorganisms. An ideal drug target should be unique to the pathogen, thus *M. tuberculosis* serine acetyltransferase is regarded as a potential drug target (10,11).

The CysE protein has been purified and characterized from certain bacteria, such as *Escherichia coli* (6,12,13), *Salmonella typhimurium* (5,14) and *Haemophilus influenzae* (15). Bioinformatic analyses have shown that *M. tuberculosis* Rv2335 is homologous to *E. coli* CysE, *S. typhimurium* CysE and *H. influenzae* CysE. Therefore, *M. tuberculosis* Rv2335 (GenBank accession no. CAB06152.1) could be a *cys*E gene that encodes the CysE protein.

In this study, we cloned and expressed the *M. tuberculosis cys*E (Rv2335) gene in *E. coli* and characterized the purified *M. tuberculosis* CysE protein. The kinetic studies on *M. tuberculosis* CysE allow for the screening of its inhibitors in the development of anti-TB drugs.

Materials and methods

Microorganisms and plasmids. E. coli NovaBlue and *E. coli* BL21 (DE3) (Novagen) were maintained as the hosts for cloning and expression, respectively. The cloning plasmid pMD18-T (Takara) with the ampicillin resistance gene was utilized to clone and sequence the target gene or DNA fragment. The expression vector pET29b (Novagen) carrying the kanamycin resistance gene was used for gene expression in *E. coli*. *M. tuberculosis* H37Rv genomic DNA was supplied by Colorado State University via an NIH contract.

Cloning the cysE (Rv2335) gene from M. tuberculosis H37Rv genomic DNA. The *M. tuberculosis cys*E gene was amplified

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Abbreviations: DTNB, 5,5'-dithio-bis-(2-nitrobenzoic acid); EDTA, ethylendiaminetetraacetic acid; IPTG, isopropyl β-D-thiogalacto-pyranoside; PMSF, phenymethylsulfonyl fluoride; NIH, National Institutes of Health

Key words: Mycobacterium tuberculosis, L-cysteine biosynthesis, serine acetyltransferase, CysE, Rv2335, kinetic parameters

Table I. Specific activity and kinetic parameters of *M. tuberculosis* CysE.

	Specific activity (μmol·min^{-1}·mg^{-1})	V_{max} (mM·min^{-1})	K_{AcCoA} (mM)	K_{ser} (mM)	K_{cat} (sec^{-1})
M. tuberculosis CysE	10.66±0.44	0.0073±0.0005	0.0513±0.0050	0.0264±0.0006	81.36±5.22

The activity was determined under specific conditions. The K_m and V_{max} of CysE were determined under the optimal conditions by double reciprocal plot. CysE, serine acetyltransferase. V_{max}, maximum velocity; K_{ser}, K_m for L-serine; K_{AcCoA}, K_m for acetyl-CoA.

from *M. tuberculosis* H37Rv genomic DNA using the following set of primers: *cys*E forward, 5'-AACATATGCT GACGGCCATGCGGG-3' (underlined sequence is the *Nde*I site) and *cys*E reverse primer, 5'-AACTCGAGGATCGAG AAGTCCTCGCCG-3' (underlined sequence is the *Xho*I site). The amplified PCR product was ligated into pMD18-T to generate the plasmid pMD18-*cys*E, which was transformed into *E. coli* NovaBlue. The positive recombinant plasmid pMD18-*cys*E was confirmed by digestion with restriction endonucleases (*Eco*RI) and subsequently sequenced. The *cys*E gene was subcloned into the *Nde*I and *Xho*I sites of pET29b, yielding the expression vector pET29b-*cys*E.

Expression, purification and identification of CysE protein. The plasmid pET29b-*cys*E was transformed into *E. coli* BL21 (DE3). BL21 (DE3)/pET29b-*cys*E culture was induced with 1 mM IPTG at 37˚C for 3 h. The cells were harvested and suspended in lysis buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 25 mM $MgCl_2$, 5% (v/v) glycerol, 1 mM EDTA, 1 mM β-mercaptoethanol and 1 mM PMSF). The cells were homogenized by sonication and the cell lysate was centrifuged at 20,000 x g for 20 min. The supernatant was then loaded onto a 1-ml Ni-NTA agarose column (Qiagen). The column was then washed with 20 ml of wash buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 20% glycerol, 60 mM imidazole and 1 mM PMSF), and the CysE protein with a His-tag at its C-terminus was eluted with 10 ml of elution buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 20% glycerol, 300 mM imidazole and 1 mM PMSF) and examined by SDS-PAGE and western blotting. The purified CysE protein was further confirmed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) (BIG, China).

Enzyme assays. The serine acetyltransferase activity of the CysE protein was determined by monitoring the increase in the absorbance of Ellman's reagent (DTNB) due to its reaction with CoA (16,17). Briefly, a 50-μl reaction mixture (50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 0.4 mM AcCoA, 2 mM L-Ser and 0.037 μg purified CysE protein) in a 96-well microtiter plate was incubated at 37˚C for 20 min. A blank control without L-Ser and AcCoA, and a positive control containing standard CoA (0.2 mM) only were included. The reaction was terminated with 50 μl of stop solution (50 mM Tris-HCl pH 7.5, 6 M guanidine hydrochloride). Fifty microliters of Ellman's reagent (50 mM Tris-HCl pH 7.5, 0.2 mM DTNB and 1 mM EDTA) was added to the reaction mixture. The mixture was incubated at room temperature for 10 min. The absorbance values were obtained using a microplate reader (Multiskan Ascent; Thermo Scientific) at a wavelength of 405 nm (18). One unit of specific enzyme activity was defined as 1 μmol of CoA-SH produced by 1 mg protein/min under specific conditions.

Characterization of M. tuberculosis CysE. The maximum velocity (V_{max}) and Michaelis constant (K_m) of *M. tuberculosis* CysE were measured by a colorimetric assay coupled with DTNB. Based on the concentration curves and time-course curves of CysE, the range of CysE initial velocities was measured. The concentration curves of CysE were plotted by measuring the reaction velocities at varying CysE concentrations and reaction times. The reactions were performed in 50 mM Tris-HCl buffer (pH 7.5) containing AcCoA, L-Ser and different concentrations of purified CysE (0.74, 1.48, 2.22, 2.96 and 3.70 μg/ml) at 37˚C for 5, 15 and 25 min. The time-course curves were plotted by measuring the amount of CoA at different reaction times (5, 10, 15, 20 and 25 min) and different concentrations of CysE (0.74, 2.22 and 3.70 μg/ml) at 37˚C. To further characterize the CysE, the effect of pH, temperature, and Mg^{2+} concentration on CysE were evaluated by measuring CysE activity in different pH buffers (3-11), at various temperatures (16-80˚C) and concentrations of Mg^{2+} (0-20 mM), respectively.

In dual-substrate reactions, the steady-state kinetic parameters K_m and V_{max} were calculated by double reciprocal plots prepared by varying the concentration of one substrate while the second substrate was in excess under optimal conditions.

Results

Cloning of the M. tuberculosis cysE gene. The PCR product for the *cys*E gene was obtained from the genomic DNA of *M. tuberculosis* H37Rv (Fig. 1A). The size of the PCR product (*cys*E gene plus *Nde*I and *Xho*I recognition sites) was 700 bp.

Expression, purification and identification of the CysE protein. The soluble *M. tuberculosis* CysE protein was expressed in *E. coli* BL21 (DE3) by induction with 1 mM IPTG. The purified CysE protein was detected by SDS-PAGE (Fig. 1B) and western blotting (Fig. 1C). The band of the CysE protein appeared at 30 kDa, which was higher than the theoretical molecular mass (24.6 kDa) of the CysE protein. The purified CysE protein was further confirmed by MALDI-TOF-MS analysis (data not shown).

Serine acetyltransferase activity of M. tuberculosis CysE protein. The serine acetyltransferase activity of *M. tuberculosis* CysE protein was detected. The specific activity of the serine acetyltransferase was 10.66±0.44 μmol/min/mg (Table I).

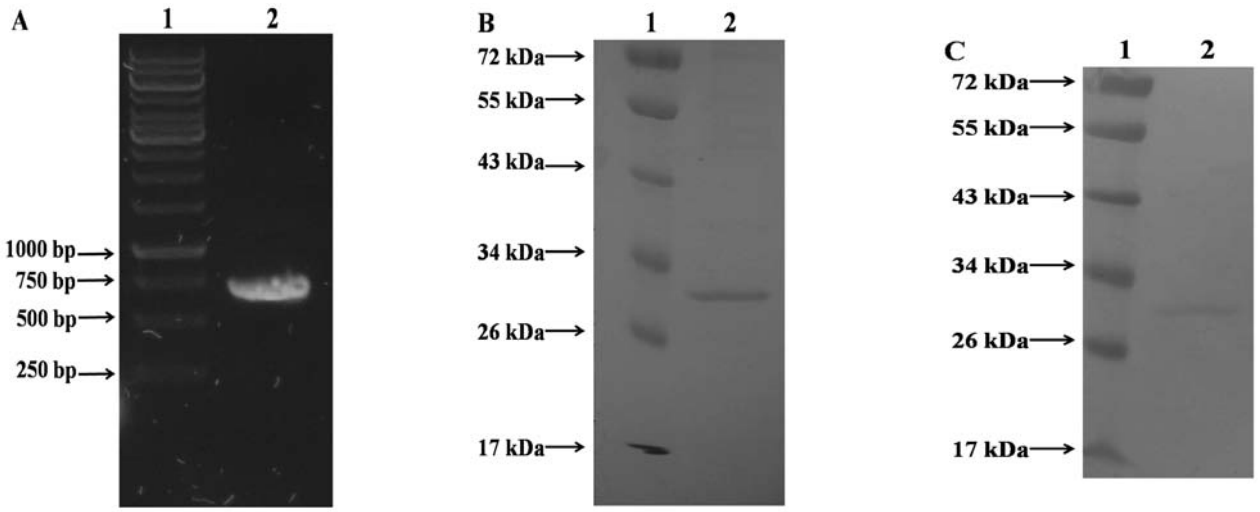

Figure 1. Analysis of *cys*E PCR products and purified *M. tuberculosis* CysE protein. (A) Electrophoretic analysis of *cys*E PCR product by 1% agarose. Lane 1, GeneRuler 1 kb DNA ladder (Fermentas); lane 2, PCR product of *cys*E gene amplified from *M. tuberculosis* H37Rv genomic DNA. (B) SDS-PAGE analysis of the purified CysE protein. Lane 1, PageRuler pre-stained protein ladder (Fermentas); lane 2, the purified CysE protein. (C) Western blotting analysis of purified CysE protein. Lane 1, PageRuler pre-stained protein ladder; lane 2, the purified CysE protein.

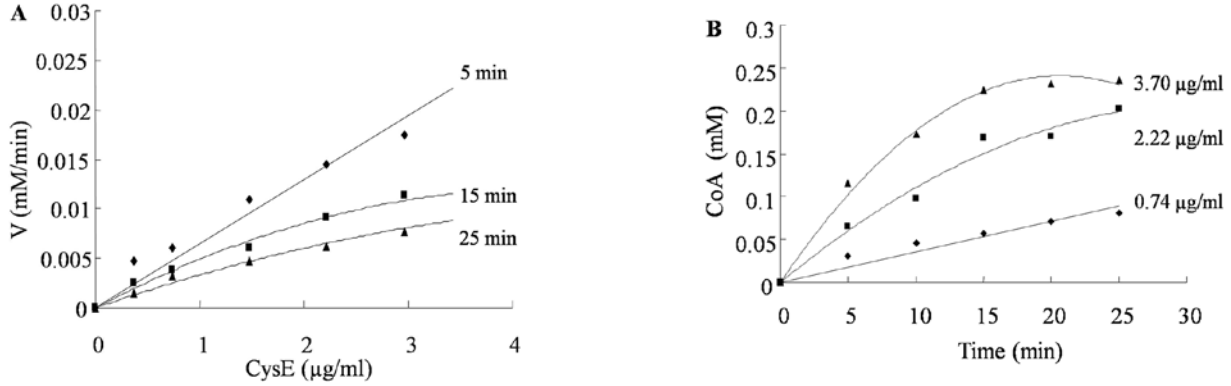

Figure 2. (A) CysE concentration curves and (B) time-course curves. (A) Reaction velocity vs. concentration of CysE at different reaction times: ◆, 5 min; ■, 15 min; ▲, 25 min. (B) Concentration of CoA vs. reaction time at different concentrations of CysE: ◆, 0.74 μg/ml; ■, 2.22 μg/ml; ▲, 3.70 μg/ml.

Characterization of M. tuberculosis CysE. The reaction velocity was proportional to the concentration of *M. tuberculosis* CysE when the reaction time was 5 min (Fig. 2A). At 15 or 25 min reaction times, the reaction velocity gradually slowed and became non-linear with the CysE concentration. Therefore, the initial velocity of CysE was within 5 min.

Within a maximum concentration limit of 0.74 μg/ml, the concentration of CoA was proportional to the reaction time (Fig. 2B). As the CysE concentration reached 2.22 or 3.70 μg/ml, the rate of CoA formation gradually decreased with reaction time. The optimal concentration for characterizing CysE was 0.74 μg/ml.

The CysE activity was determined at varying pHs with appropriate buffer systems (3-11) after the initial velocity and optimal CysE protein concentration were set (Fig. 3A). The optimal pH for CysE was 7.5. The optimal temperature for CysE was investigated from 16 to 80˚C (Fig. 3B), with the highest activity observed as the temperature reached 37˚C. The catalytic activity of CysE was not significantly changed by varying the Mg^{2+} concentration (Fig. 3C), indicating that Mg^{2+} had no effect on the CysE activity.

The steady-state kinetic constants were determined under the optimal conditions and the initial velocity by a double reciprocal plot (Fig. 4). The V_{max} value of CysE was 0.0073±0.0005 mM/min. The K_m of CysE against AcCoA was 0.0513±0.0050 mM, while the K_m value of L-Ser was 0.0264±0.0006 mM (Table I).

Discussion

Serine acetyltransferase is an enzyme involved in cysteine biosynthesis, and it plays an important role in the growth of *M. tuberculosis* (10). In addition, this enzyme only exists in microorganisms and plants (9), making serine acetyltransferase a potential anti-TB drug target.

M. tuberculosis Rv2335 is predicted to be a *cys*E gene encoding serine acetyltransferase. Bioinformatic analyses have shown that the *M. tuberculosis* Rv2335 protein is 45% identical to *E. coli* CysE, *S. typhimurium* CysE and *H. influenzae* CysE using the Basic Local Alignment Search Tool (BLAST). Serine acetyltransferase is a member of the hexapeptide acetyltransferase family (19). This protein family has

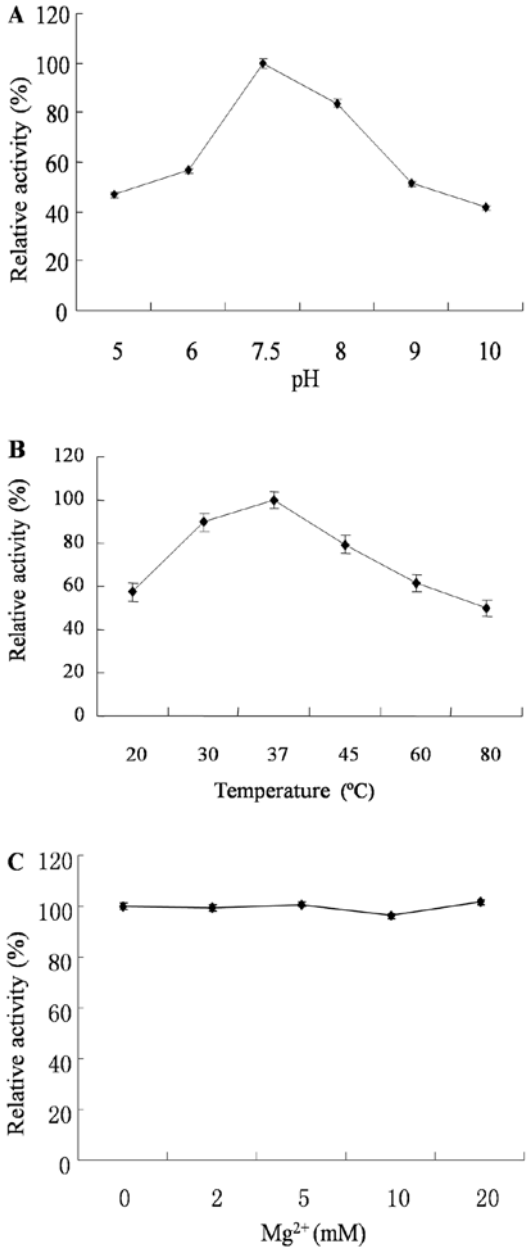

Figure 3. Determination of the optimal conditions for *M. tuberculosis* CysE activity. (A) pH curve; (B) temperature curve; (C) Mg^{2+} concentration curve.

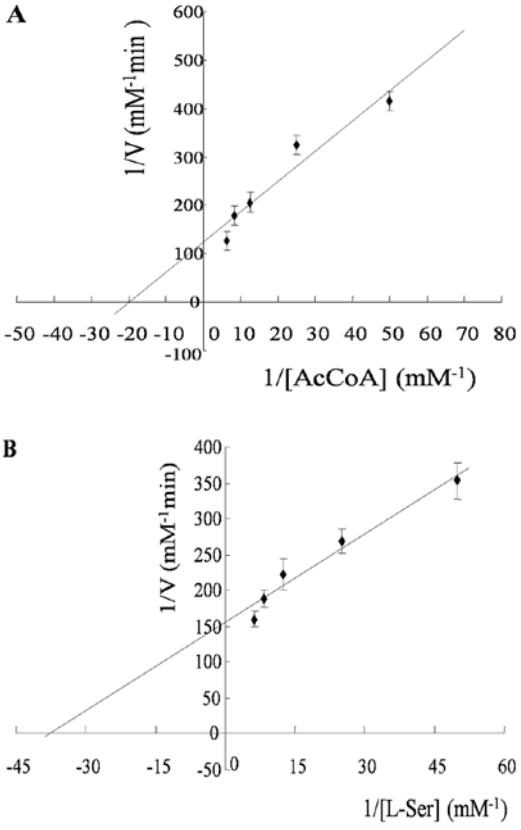

Figure 4. Determination of V_{max} and K_m values for *M. tuberculosis* CysE. (A) V_{max} and K_m values for AcCoA from a double reciprocal plot; (B) V_{max} and K_m values for L-Ser from a double reciprocal plot.

a conserved active left-handed-β-helix (LβH) domain, which is composed of a six-peptide ([LIV]-[GAED]-X2[STAV]-X) tandem repeat (15,20,21). The *M. tuberculosis* Rv2335 protein contained the tandem repeat and showed LβH structure when modeled using the NCBI Conserved Domain Search (data not shown).

To identify the function of *M. tuberculosis* CysE, the *M. tuberculosis cys*E (Rv2335) gene was amplified with high fidelity DNA polymerase, and the soluble CysE protein was expressed in *E. coli*. SDS-PAGE and western blotting showed that the molecular weight of the expressed CysE protein (~30 kDa) was higher than predicted. This finding could be due to the auxiliary fusion of six histidines to the recombinant *M. tuberculosis* CysE protein generated from the pET29b vector. The six consecutive histidines impart a strong positive charge that may retard the mobility of the CysE protein in SDS-PAGE.

As indicated in Table I, *M. tuberculosis* CysE demonstrated serine acetyltransferase activity of 10.66 μmol/min/mg. The specific activity of *E. coli* serine acetyltransferase has been reported as 71.6 μmol/min/mg (22). The specific activity of *M. tuberculosis* CysE is lower than that of *E. coli* CysE, possibly because of the different methods of purification. *M. tuberculosis* CysE exhibited its highest acetyltransferase activity at pH 7.5 and 37˚C. The optimal pH is consistent with those reported for other bacteria, but the optimal temperature is different from those reported for other bacteria such as *S. typhimurium* (25˚C) (14), *E. coli* (25˚C) (12) and *H. influenzae* (25˚C) (15). The K_m for L-serine (K_{ser}) of *M. tuberculosis* CysE (0.026 mM) is lower than the K_{ser} of *S. typhimurium* CysE (0.7 mM) and *E. coli* CysE (1.17 mM) (12,23). The K_m for AcCoA (K_{AcCoA}) of *M. tuberculosis* CysE (0.051 mM) is also lower than that of *S. typhimurium* CysE (0.1 mM) and *E. coli* CysE (0.2 mM) (12,23). In the present study, the K_{AcCoA} of *M. tuberculosis* CysE was 0.051 mM, while the K_{ser} was 0.026 mM. This finding suggests that CysE had higher affinity for L-Ser than AcCoA, and CysE was bound more easily to L-Ser than to AcCoA in *M. tuberculosis*. Cysteine is reported to inhibit the activity of serine acetyltransferase in its biosynthetic pathway by a feedback mechanism (7,12,15). Furthermore, cysteine was found to bind *E. coli* CysE at the serine substrate site rather than at the acetyl-CoA substrate site from the structural study on acetyltransferase (20). This finding indicates that it is preferable to screen and design

compounds against the L-serine site to inhibit the activity of CysE.

In summary, serine acetyltransferase CysE was encoded by the *cys*E (Rv2335) gene in *M. tuberculosis*. We investigated the kinetic parameters and optimal catalytic conditions of CysE using simple and rapid enzyme assays. The CysE assay and kinetic properties of CysE will facilitate the high-throughput screening of inhibitors against CysE. However, there are currently no reports of the crystal structure and active sites of *M. tuberculosis* CysE. The expressed soluble CysE protein will be available to further elucidate its crystal structure and active sites.

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