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Cloning, expression, purification and bioinformatic analysis of 2-methylcitrate synthase from *Mycobacterium tuberculosis*

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ABSTRACT

Objective: To clone, express and purify 2-methylcitrate synthase (*Rv1131*) gene of *Mycobacterium tuberculosis* (*M. tuberculosis*) and to study its structural characteristics using various bioinformatics tools. **Methods:** *Rv1131* gene was amplified by polymerase chain reaction using *M. tuberculosis* H37Rv genomic DNA and cloned into pGEM-T easy vector and sequenced. The gene was sub-cloned in pET28c vector, expressed in *Escherichia coli* BL21 (*E. coli* BL21) (DE3) cells and the recombinant protein was identified by Western blotting. The protein was purified using Nickel affinity chromatography and the structural characteristics like sub-cellular localization, presence of transmembrane helices and secondary structure of the protein were predicted by bioinformatics tools. Tertiary structure of the protein and phylogenetic analysis was also established by *in silico* analysis. **Results:** The expression of the recombinant protein (Rv1131) was confirmed by western blotting using anti-HIS antibodies and the protein was purified from the soluble fraction. *In silico* analysis showed that the protein contains no signal peptide and transmembrane helices. Active site prediction showed that the protein has histidine and aspartic acid residues at 242, 281 & 332 positions respectively. Phylogenetic analysis showed 100% homology with major mycobacterial species. Secondary structure predicts 2-methylcitrate synthase contain 51.9% alpha-helix, 8.7% extended strand and 39.4% random coils. Tertiary structure of the protein was also established. **Conclusions:** The enzyme 2-methylcitrate synthase from *M. tuberculosis* H37Rv has been successfully expressed and purified. The purified protein will further be utilized to develop assay methods for screening new inhibitors.

1. Introduction

Drug resistance in *Mycobacterium tuberculosis* (*M. tuberculosis*) has become a global problem in recent years and the emergence of multi drug resistant strains has dramatically increased the mortality worldwide. No tuberculosis-specific drug has been discovered for more than four decades. *Mycobacterium bovis* BCG (*M. bovis* BCG), an anti-TB vaccine, developed by Calmette and Guerin, has been used worldwide till date. But its protective efficacy against the pathogen varies from individual to individual. During infection, *M. tuberculosis* relies heavily on fatty acid metabolism for both energy supply and for synthesis of essential biomolecules required for its growth and survival in the living host[1-3]. Propionyl-CoA generated along with acetyl-CoA during the breakdown of odd-chain fatty acids and amino acids are reported to be potentially toxic to the mycobacteria[4,5]. In mycobacteria, propionyl-CoA metabolism is linked to synthesis of essential lipid virulence factors like phthiocerol dimycocerosate (PDIM) and sulfolipid-1 (SL-1)[6]. *M. tuberculosis* metabolizes propionyl-CoA through methylcitrate cycle. Mycobacterial methylcitrate cycle consists of 2-methylcitrate synthase (Rv1131) and methylcitrate dehydratase (Rv1130) but do not contain a dedicated methylisocitrate lyase (MCL)[7,8].

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Isocitrate lyase of glyoxylate pathway does MCL activity by its capability to accommodate both the substrates (methylisocitrate and isocitrate) in its active site[9]. 2-methylcitrate synthase catalyzes the condensation of propionyl-CoA with oxaloacetate to form 2-methylcitrate. Accumulation of 2-methylcitrate inside the cells is reported to inhibit the bacterial cell growth and blocks gluconeogenesis[10,11]. Enzymes involved in methylcitrate cycle do not have homologue in humans and thus considered as potential target for new anti-tubercular drug development. To study the function of 2-methylcitrate synthase enzyme, we expressed the protein in *Escherichia coli* BL21 (*E. coli* BL21) (DE3) and purified using Ni-NTA affinity chromatography. Physical and structural characteristics of the purified protein were predicted using various bioinformatics tools.

2. Materials and methods

2.1. Bacterial strains, plasmids and chemicals

E. coli DH5 α was used for general cloning procedures and *E. coli* BL21 (DE3) was used for expression studies. *M. tuberculosis* H37Rv genomic DNA was purchased from TCGA, New Delhi. T4 DNA ligase, restriction/ DNA modifying enzymes were obtained from New England Biolabs (USA). Oligonucleotide primers were synthesized from Sigma, Inc. (USA). Plasmid pET28c was purchased from Novagen (Germany). Ni-NTA agarose and other PCR purification/ Plasmid isolation kits were obtained from Qiagen (Germany). Phenylmethanesulfonylfluoride (PMSF), lysozyme and all analytical grade chemicals were either from Himedia chemicals (India). Standard recombinant DNA techniques used were as described elsewhere[17].

2.2. PCR amplification of Rv1131

The nucleotide sequence of *Rv1131* gene was retrieved from NCBI (http:// www.ncbi.nlm.nih.gov) for designing gene specific primers. PCR was performed using *M. tuberculosis* H37Rv genomic DNA as template (DMSO was added to the PCR reaction mixture to lower the melting temperature since *M. tuberculosis* H37Rv Genomic DNA is GC rich template). *Rv1131* gene was amplified using gene-specific primers (Table 1) containing *Bam*H Ⅰ (Forward primer) and *Hind* Ⅲ (Reverse primer) restriction sites. Phusion®High-Fidelity DNA Polymerase was used to amplify the gene under following conditions: 95 ℃ for 5 min, 25 cycles (95 ℃ 1 min; 55 ℃ 1 min; 72 ℃ 1 min), and final extension of 72 ℃ for 10 min. The PCR products were analyzed on a 1.2% agarose gel stained with ethidium bromide.

Table 1

Primers used in the study.

Primer name	Sequence details (5′- 3′) **
Rv1131 forward	ATTCCACCAGGATCCTTTCGATG
Rv1131 reverse	GAGAAAGCTTATGGCCCATAAGAG
T7 promoter	TAATACGACTCACTATAGGG
T7 terminator	CTAGTTATTGCTCAGCGGTG

** Restriction sites in the primer have been underlined.

2.3. Cloning of Rv1131 gene in E. coli

PCR products were gel purified and cloned into pGEM-T easy vector and sequenced. The plasmid containing *Rv1131* gene was digested with *Bam*H Ⅰ and *Hind* Ⅲ, restriction enzymes and the digested gene product was gel purified and ligated into expression vector pET28c (digested with the same enzymes) overnight at 16 ℃. The ligated products were then transformed into *E. coli* DH5 α cells using standard protocol. Colonies were screened by PCR using T7 Promoter and T7 Terminator primers (Table 1) and the recombinant plasmid was isolated from the positive colonies by alkaline lysis method. The recombinant plasmid was then subjected to restriction analysis using *Bam*H Ⅰ and *Hind* Ⅲ restriction enzymes.

2.4. Expression of Rv1131 gene in E. coli

The recombinant plasmid pET28c-Rv1131 was transformed into *E. coli* BL21 (DE3) cells for expression studies. A single colony of *E. coli* BL21 (DE3) containing pET28c-Rv1131 was inoculated in 5 mL of LB medium containing kanamycin (50 μg/mL) and grown at 37 ℃ overnight. One mL of this culture was added in 100 mL fresh LB medium containing kanamycin (50 μg/mL) at 37 ℃ until A_{600} 0.6 was reached. To the growing culture of *E. coli* BL21 (DE3) containing pET28c-Rv1131, IPTG was added to a final concentration of 1 mM and incubated at 30 ℃ for 4 h. After induction, cells were harvested by centrifugation at 8 000 *g* for 10 min and the total proteins of the control and induced cells were analyzed by 12% SDS PAGE. The proteins were visualized by staining the gel with Coomasie blue (0.05%) for 30 min in a rocking shaker.

2.5. Purification and Western Blotting

E. coli BL21 (DE3) cells transformed with pET28c-Rv1131 were grown at 37 ℃ in LB broth containing kanamycin (50 μg/mL) to an A_{600} of 0.6 and the expression was induced by the addition of 1 mM IPTG for 4 h at 30 ℃. Cells from 500 mL culture were resuspended in 5 mL of buffer A (20 mM tris-HCL, pH 8.0, 500 mM NaCl, 30 mM imidazole, 1 mM PMSF and 10 μg/mL Lysozyme). After one cycle of freeze-thaw, cells were lysed by sonication (30 s pulse × 30 s pause). The soluble fraction was separated by centrifugation at 15 000 rpm for 30 min at 4 ℃ and loaded

on to a pre-equilibrated 2 mL (bed volume) Ni-NTA affinity column. After washing the column with 30 bed volumes of buffer B (50 mM tris-Cl, 500 mM NaCl, 40 mM imidazole, pH 8.0) at a flow rate of 1.0 mL/min, the bound protein was eluted in buffer C (50 mM tris-Cl, 300 mM NaCl and 200 mM imidazole, pH 8.0). The purified proteins were quantified using Bradford method. For western blotting, the purified protein was electrophoresed on SDS-PAGE (12%) and transferred to PVDF membrane. After blocking, the membrane with Bovine serum albumin (5%) for 90 min, the membrane was probed with 1:5 000 dilution of anti-HIS mouse IgG antibody followed by HRP-conjugated goat anti-IgG antibody. The blot was developed using 3, 3′-Diaminobenzidine substrate.

2.6. In silico analysis of Rv1131 gene and 2-methylcitrate synthase protein

The nucleotide and protein sequences analyzed by *in silico* methods were retrieved from NCBI database (http:// www.ncbi.nlm.nih.gov). The multiple sequence alignment was carried out using ClustalW Program (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and the alignment file was used to generate phylogenetic relationship using MEGA 5.1Beta software. Signal peptides and subcellular localization was predicted by SignalP (http://www.cbs.dtu.dk/services/SignalP/) and TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/) program respectively. Secondary structure of the protein was predicted using PredictProtein Program (https://www.predictprotein.org/). Tertiary structure of the protein was established using SWISS-MODEL server (http://swissmodel.expasy.org/) based on homology modeling and analysed by Rasmol software (http://rasmol.org/OpenRasMol.html).

3. Results

3.1. Cloning of Rv1131 gene

Rv1131 gene was amplified by PCR (Figure 1A). A 1.2 kb DNA fragment containing an open reading frame of 393 amino acids was obtained. The gene was then cloned into pGEM-T easy vector and screened by blue/white screening method. The sequence of positive recombinant plasmid was verified by DNA sequencing. The positive recombinant plasmid was digested with *Bam*H Ⅰ and *Hind* Ⅲ restriction enzymes and the gene product was gel purified and ligated with pET28c vector (digested with the same enzymes) and transformed into *E. coli* DH5 α . Colonies were screened by PCR using T7 promoter and T7 terminator primers (Table 1) to identify recombinant colonies. Recombinant plasmid was isolated from the positive colony and subjected to restriction analysis using *Bam*H Ⅰ and *Hind* Ⅲ, restriction enzymes (Figure 1B). The pET28c vector was chosen because it contains *N*-terminal 6×His *Tag* which facilitates purification by Nickel affinity chromatography.

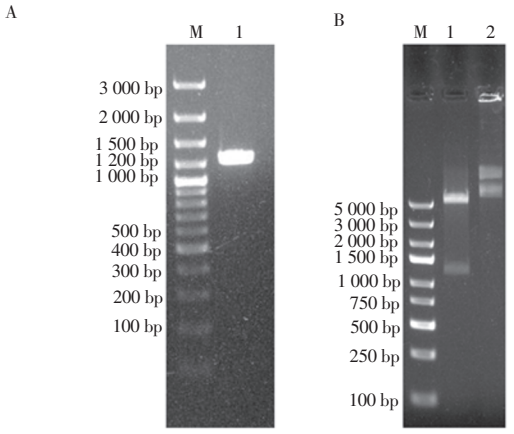

Figure 1. PCR amplification of *Rv1131* gene and restriction endonuclease analysis of recombinant pET28c-Rv1131.
(A) *Rv1131* gene amplified by PCR using *M. tuberculosis* H37Rv genomic DNA. Lane M, 100 bp DNA Ladder; lane 1, Rv1131 PCR product.
(B) Recombinant plasmid pET28c-Rv1131 was identified by restriction analysis using *Bam*H Ⅰ and *Hind* Ⅲ enzymes. Lane M, 1 kb DNA Ladder; Lane 1, pET28c-Rv1131 digested with *Bam*H Ⅰ and *Hind* Ⅲ; Lane 2, undigested pEt28c-Rv131 plasmid.

3.2. Expression of 2-methylcitrate synthase

E. coli BL21 (DE3) was transformed with pET28c-Rv1131 recombinant plasmid for 2-methylcitrate synthase production. Expression of 2-methylcitrate synthase was induced by adding IPTG to a final concentration of 1 mM at lower incubation temperature (30 ℃) for 4 h. The induced protein was observed by analyzing the IPTG added cells along with control cells (IPTG not added) by 12% SDS-PAGE stained with Coomasie blue G250 and as expected a band at 43 kDa was observed (Figure 2A). The solubility and cellular localization of the protein was analyzed by sonication of the cell pellet and subjecting the soluble and insoluble fractions to SDS-PAGE. 2-methylcitrate synthase was found to be present predominantly in the soluble fraction. Nickel affinity chromatography was used for purifying the protein from the soluble fraction.

3.3. Purification and identification of 2-methylcitrate synthase

The 2-methylcitrate synthase was purified from the soluble fraction. The supernatant soluble fraction was incubated with Ni-NTA agarose matrix overnight at 4 ℃ and then loaded into the column. After washing the column with wash buffer, the bound proteins were eluted using elution buffer containing imidazole. The eluted fractions were then analyzed by SDS-PAGE. The analysis showed a single band at 43 kDa in the gel (Figure 2B). The eluted protein was confirmed by western blotting using anti-His IgG antibodies. The eluted fractions were subjected to SDS PAGE and transferred to PVDF membrane and probed using anti-His IgG antibodies. The blot was developed using 3,3′-diaminobenzidine and it showed a single band at 43 kDa (Figure 2C).

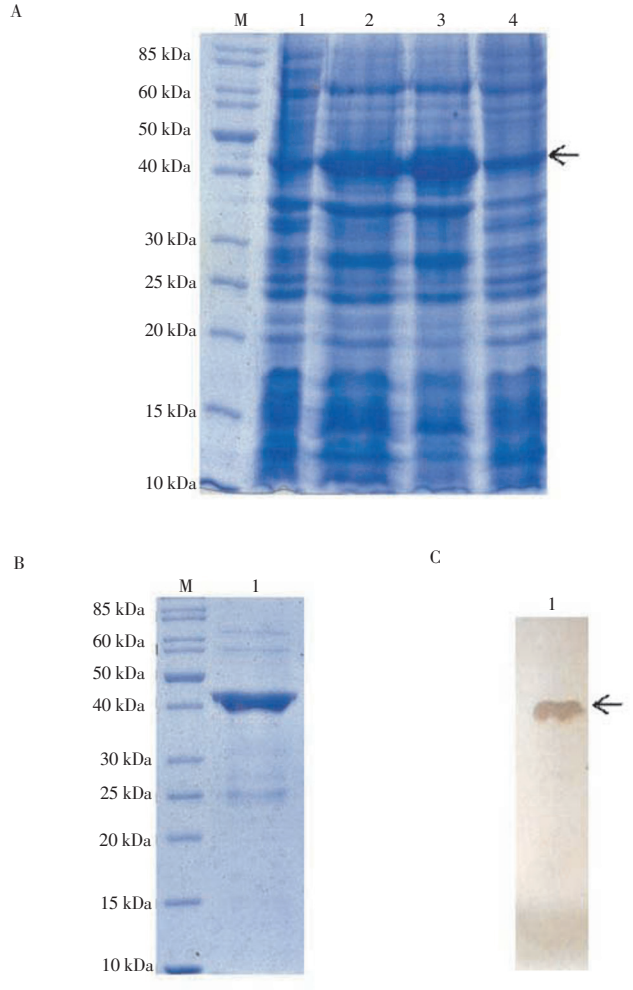

Figure 2. SDS-PAGE analysis and Western blotting identification of 2-methylcitrate synthase protein expressed in *E. coli* BL21 (DE3) cells.
(A) SDS-PAGE analysis of total proteins, soluble and insoluble fractions expressed. Lane M, protein molecular mass standards; lane 1, cell lysate of bacteria transformed with pET28c-Rv1131 without induction; lane 2, cell lysate of bacteria transformed with pET28c-Rv1131 with IPTG (1 mM) induction for 4 h; lane 3, induced supernatant fraction; lane 4, induced pellet fraction.
(B) SDS-PAGE analysis of purified 2-methylcitrate synthase protein. Lane M, protein molecular mass standards; lane 2, the protein purified from the supernatant fraction.
(C)Western blotting analysis of *N*-terminal 6×His tagged 2-methylcitrate synthase protein. Lane 1, *N*-terminal 6×His tagged 2-methylcitrate synthase protein. The arrows indicates the location of recombinant protein.

3.4. Analysis of structural characteristics of 2-methylcitrtae synthase by in silico methods

2-methylcitrate synthase consists of 393 amino acids with a theoretical molecular mass of 42.9 kDa and pI value of 8.61. Sequence alignment reveals 2-methylcitrate synthase of *M. tuberculosis* H37Rv to share 100% homology with *M. tuberculosis* H37Ra and *M. tuberculosis* CDC151 and shows 70% with other mycobacterial species (Figure 3). 2-methylcitrate synthase does not show the presence of signal peptide, transmembrane helices and was found to be located outside the membrane (Figure 4). An *N*-glycosylation site and an *N*-myristoylation site also have been predicted in the protein. Secondary structure prediction showed that the protein has 51.9% alpha-helix, 8.7% extended strand and 39.4% random coils. The protein was also predicted to have Protein kinase C phosphorylation site at 53, 90, 96, 293, 303 residues respectively and Casein kinase Ⅱ phosphorylation sites at 21, 82, 90, 195, 222, 303, residues respectively. The tertiary structure of 2-methylcitrate synthase protein was also established based on homology modeling (Figure 5).

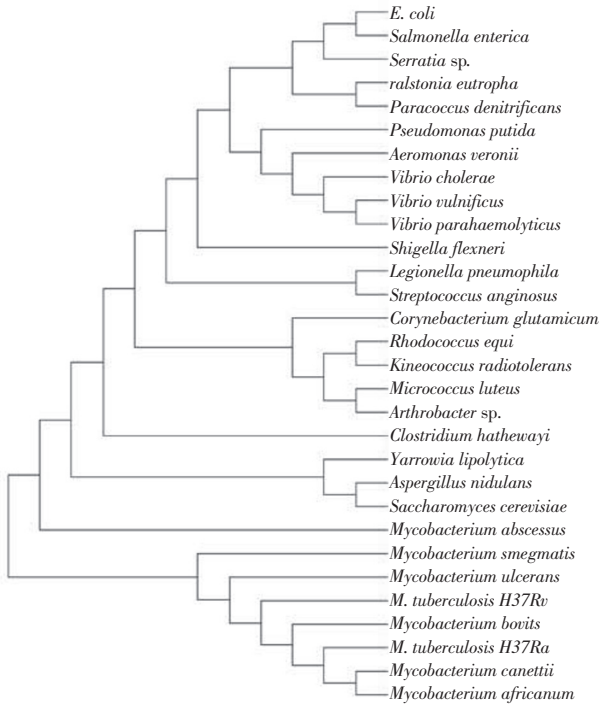

Figure 3. Evolutionary tree analysis of 2-methylcitrate synthase from other microorganisms.

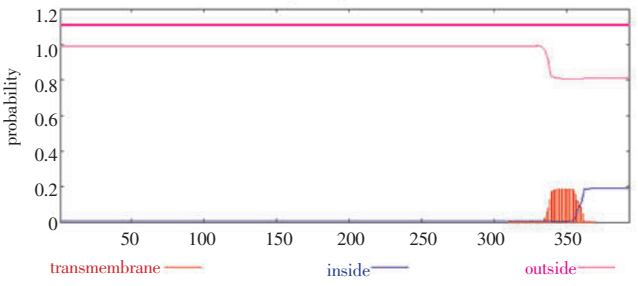

Figure 4. Sub-cellular localization and transmembrane helices prediction of 2-methylcitrate synthase.

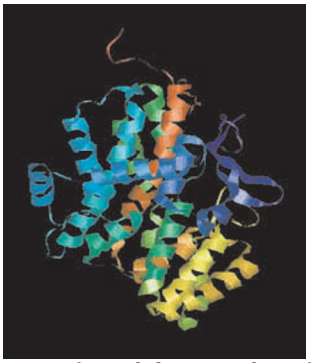

Figure 5. Tertiary structure of 2-methylcitrate synthase of *M. tuberculosis*.

4. Discussion

The genome of *Mycobacteria* consists of significant number of open reading frames (ORFs) that encode proteins that play a role in the growth or survival of the bacterium inside macrophages[7,12]. Multidrug-resistant strains are on the rise, and its infection is often associated with HIV infection. Mycobacterial persistence inside the host macrophages remains the biggest challenge for developing new drugs against the pathogen. So far, the drugs which are available in the treatment of tuberculosis target only the actively growing bacteria. Metabolic adaptations are required for long term persistence of mycobacteria inside the host. *M. tuberculosis* shifts its energy source from glucose to fatty acids because it has the ability to induce formation of foamy macrophages which are loaded with large number of lipid-containing bodies[13]. Methylcitrate cycle, which enables *M. tuberculosis* to utilize fatty acids, has been identified as potential metabolic pathway that could be targeted by new drugs[14,15]. Methylcitrate cycle is also known to be an important pathway for anaplerotic replenishment of tricarboxylic acid cycle intermediates and for gluconeogenesis in most of the cells[16]. In *M. tuberculosis*, it has been reported that tricarboxylic acid (TCA) intermediates are critical for infection[16]. The focus of this study is to identify potential new drug targets in *M. tuberculosis*. 2-methylcitrate synthase enzyme is one of the key enzymes of the most common propionyl-CoA metabolizing pathway in micro-organisms and is a conserved enzyme. Targeting this enzyme therefore could have multiple effects in *M. tuberculosis*: build-up of toxic propionyl CoA, inhibition of gluconeogenesis and on the overall efficiency of TCA because of its role in anaplerotic replenishment of the TCA intermediates.

Through bioinformatics tools its localization, modification sites and possible active sites residues have been identified which could enable researchers to develop new anti-tuberculosis drugs by targeting 2-methylcitrate synthase enzyme.

Conflict of interest statement

We declare that we have no conflict of interest.

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