

Original Research Article

<https://doi.org/10.20546/ijcmas.2019.809.254>

## *Mycobacterium tuberculosis* tlyA E59Q is Stable Compared to the Native Protein

V. B. Shivaleela<sup>1</sup>, ShaikKalimulla Niazi<sup>2</sup>, P. Sharada<sup>1\*</sup> and K. Giri Gowda<sup>3\*</sup>

<sup>1</sup>Department of Biotechnology, Basaveshwara Engineering College,  
Bagalkot-587102, Karnataka, India

<sup>2</sup>Department of Biotechnology, Preparatory Health Sciences, Riyadh Elm University,  
Riyadh, Saudi Arabia

<sup>3</sup>Sampoorna International Institute of Agri.Science & Horticulture Technology,  
Mandya-571433, Karnataka, India

\*Corresponding author

### ABSTRACT

#### Keywords

tlyA,  
Mycobacterium  
tuberculosis,  
methyltransferase,  
antibiotic  
resistance,  
mutation.

#### Article Info

Accepted:  
22 August 2019  
Available Online:  
10 September 2019

The causative agent of human tuberculosis disease, *mycobacterium tuberculosis* (*Mtb*), infects one-third of the world population. *Mycobacterium tuberculosis* tlyA is an important protein and the mutation can cause resistance to antibiotics. Understanding the structure-function of *mycobacterium tuberculosis* tlyA is essential for preventing antibiotic resistance. Structure of *Mycobacterium tuberculosis* CTD of tlyA structure was solved, whereas its biological functions are not fully understood. Here, we studied tlyA protein by mutational analysis and molecular modelling studies. Further, purified tlyA is found to be very stable compared to the native tlyA and retained its three dimensional structure. In the absence of full length tlyA structure, molecular model was generated by I-Tasser server using tlyA CTD crystal structure as a template. Generated model showed 90% of residues in the allowed region, 7% in additional allowed region and 3% residues in disallowed region. These results reveal *Mtb*-tlyA NTD is important for its structure and function and E59Q is a critical residue for retaining the N-terminus.

### Introduction

*Mycobacterium tuberculosis* (*Mtb*) is one of the most infectious pathogens and causes about 1.3 million deaths and 10 million cases

of new infections every year (WHO report, 2018). Drug resistant *Mtb* becoming more prevalent, major concern is the rising number of TB patients involving *Mtb* strains significantly increased are multidrug-resistant

including resistant to isoniazid and rifampicin (Witek *et al.*, 2017).

Understanding the modification in proteins for drug resistance is critical for the treatment of TB. Ribosome is one of the most common target so antibiotics in the cell and mutations in different components of ribosome are responsible for drug resistance. Several mutations in 23S and 16SrRNA genes, rRNA methyltransferases, and ribosomal proteins in different mycobacterial strains have been shown to be responsible for resistance to different drugs (Kumar *et al.*, 2011). rRNA methyltransferases are strong candidates for drug targeting against mycobacteria. Resistance to antibiotics in *Mtb* can acquire via mutation of *tlyA*, the gene encoding the protein *tlyA* (Rv1694), a proposed virulence factor. *tlyA* belongs to a unique group of methyltransferases for which the loss of function confers bacterial antibiotic resistance. Many bacterial genera lack *tlyA*, the potent antibiotic activity of capreomycin is specific against *Mtb*. Capreomycin targets the ribosome of *Mtb* at the interface of the small and large subunits and requires ribosomal RNA (rRNA) methylation for optimal binding and thus inhibition of ribosome function (Kumar *et al.*, 2011).

However, treatment of TB has become problematic not only because of the side effects of aminoglycosides but also because of the increased incidence of virulent, capreomycin-resistant *Mtb* strains generated by inactivation of *tlyA* (Kumar *et al.*, 2011, Maus *et al.*, 2005). Despite the critical role of *tlyA* in capreomycin sensitivity, identification of inactivating mutations that cause resistance is lacking and our current understanding of *tlyA* structure and mechanism of action remains limited.

Despite the importance of *tlyA* activity in capreomycin action and resistance, many

molecular details of the *tlyA* mechanism of action remain largely unknown. Therefore, detailed molecular studies of *tlyA* are urgently required for the better understanding of the resistance. Previous studies demonstrated that *Mtb-tlyA* folds into two stable structural domains connected by a protease-sensitive linker, with rRNA binding and SAM binding/methyltransferase activities expected to reside in the amino- and carboxyl terminal domains (NTD and CTD), respectively. Studies *also* showed the role of a minimalist N-terminal domain in recruiting Rv2966c to ribosome to carry out its methyltransferase function.

Hence, a comprehensive understanding of *tlyA* full length protein structure and its mutational studies is required for understanding the resistance in *Mtb-tlyA*. In the present study, we mutated critical residue E59Q of *tlyA* and the intact protein containing NTD and CTD was purified. Molecular model *Mtb-tlyA* was generated using (PDB ID:5EOV) as template for clear understanding the structure-function relationship.

## Materials and Methods

### Bacterial strains and plasmid

pETDuet-1 vector system was obtained from Invitrogen (California, USA) and was used according to the manufacturer's instructions. *E.coli* DH5 $\alpha$  and *E.coli*BL-21 competent cells were obtained from Invitrogen (California, USA).

### PCR amplification of *tlyA* native and E59Q

Oligonucleotide primers used for amplification of *Mtb-tlyA* native were designed based on the *tlyA* sequence of *Mycobacterium tuberculosis* strain H37Rv deposited in the genome database (NCBI accession no.AQO55200.1). The sequence of

the forward primer was 5'-GCGGAATT CGATGGCACGACGTGCCCGCGTT-3' and the reverse primer was 5'-TATGGTAC CTTACGGGGCCCTCGCTAATCGCACG-3'.

The reaction mixture used for gene amplification contained 2 µl of 5X phusion buffer supplied with the enzyme, 200 µM of each dNTPs, 0.5 µM of each primer, 150 ng of DNA template, and of 0.02 U/µl phusion DNA polymerase (New England Biolabs, Massachusetts, USA) and water to a final volume of 20 µl. After optimizing the conditions for polymerase chain reaction (PCR), the gene was successfully amplified using the following PCR conditions: 98 °C for 30 sec followed by 30 cycles of denaturation at 98 °C for 10 sec, annealing at 60 °C for 30sec, extension at 72 °C for 30sec and the final extension was carried out at 72 °C for 10 min on a PTC-100 Thermocycler (M.J. Research, Watertown, MA). The PCR product was analysed by electrophoresis on a 1% agarose gel and showed a DNA band of the expected size which was purified using a gel extraction kit (Thermofisher Scientific, Waltham, Massachusetts, USA). Mutant of *tlyAE59Q* was designed, the sequence of the forward primer was 5'-ACCGTGGTGA CCGACAGTCAACGCGCCTGGGTATCGC GC-3' and the reverse primer was 5'-GCGCGATACCCAGGCGCGTTGACTGTC GGTCACCACGGT-3'. Sequential two step PCR amplification was performed for the *tlyAE59Q* mutant using forward and reverse primers of the native *tlyA*.

### **Cloning and DNA sequencing**

Purified PCR product was cloned into plasmid DNA using the pETDuet-1 vector system (Novagen, Wisconsin, USA). The PCR product and pETDuet-1 vector was digested with restriction enzymes EcoRI and KpnI for 2h at 37° C, product was purified. 2 µl of purified PCR product was mixed with 0.5 µl linearized pET-Duet-1 cloning vector in

presence of 0.5 µl T4 DNA ligase (Thermofisher Scientific, Massachusetts, USA) and incubated overnight at 16 °C. Then the ligation mixture was directly used for the transformation of CaCl<sub>2</sub>competent DH5α cells by heat shock method (Inoue *et al.*, 1990). Transformed cells were screened by ampicillin resistant, blue-white selection with X-gal and IPTG. Colony PCR was performed to screen positive colonies. Positive colonies were picked, grown overnight in 5 ml of LB broth at 37 °C and plasmids were isolated using commercial mini-prep kit (GCC Biotech, Joychandipur, West Bengal, India). Restriction digestion screening of the isolated plasmids were done to select the construct containing the correct size insert and selected constructs were sequenced. Sequencing was performed using vector specific T7 promoter primer.

### **Expression and purification**

The pETDuet-1 plasmid containing E59Q mutant gene was transformed into *E. coli* BL21 (Star) competent cells. For protein expression, transformed BL21 (Star) cells were grown at 37°C to an optical density of 0.6 at 600 nm (OD<sub>600</sub>) and induced with 0.5 mM isopropyl-β-thiogalactopyranoside (IPTG). Induced cultures were transferred to 18° C and cells were grown for 12-14 h. Cells were harvested by centrifugation at 18,000 rpm at 4°C and cell pellets were stored at -20°C until further use. For protein purification, cell pellets from 1 litre culture were resuspended in 20 ml of ice cold binding buffer containing 50 mM TrisHCl (pH 7.5), 300 mM sodium chloride, 10% glycerol (v/v) and 5 mM β-mercaptoethanol. Lysozyme was added to a final concentration of 100 mg/ml and kept on rocking platform at 4°C for 45 min. PMSF was added immediately after the lysis (0.2 mM). Cells were disrupted by sonication on ice with 50% amplitude and a pulse of 20 sec on and 60 sec off for 15 min.

The lysate was centrifuged at 18,000 rpm for 1h at 4°C to separate supernatant from cell debris. The supernatant was loaded onto 5 ml HisTrap HP affinity column pre-equilibrated with the binding buffer. Protein was eluted by running a linear gradient of 0–1000 mM imidazole in 60 ml of buffer A [50 mM Tris HCl (pH 7.5), 1 M imidazole, 300 mM sodium chloride and 10% glycerol (v/v)] at a flow rate of 1 ml/min. Eluted fractions were analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fractions containing pure protein were pooled together. Fractions containing E59Q*tlyA* was concentrated using a 10 kDa cut-off Amicon Ultra-15 concentrator (Millipore, Bedford, Massachusetts, USA).

### **Gel filtration Chromatography**

The concentrated protein was loaded onto HiLoad 16/60 prep grade Superdex75 size-exclusion chromatography column pre-equilibrated with buffer containing 20mM Tris-HCl (pH 7.5), 1M NaCl, 10% (v/v) glycerol and 5mM  $\beta$ -mercaptoethanol using AKTA purification system (GE Healthcare). Protein was allowed to pass through the column at a rate of 0.8 ml/min. The major peak fractions containing pure protein were pooled and concentrated. Homogeneity of purified E59Q mutant *tlyA* protein was analysed on 15% SDS-PAGE. Protein concentration and yield were determined using the Bio-Rad protein assay kit with bovine serum albumin (BSA) as a standard.

### **SDS-PAGE**

SDS-PAGE was performed using 15% acrylamide gels, followed by the method of Laemmli (1970). The expressed soluble fractions were diluted with the sample buffer and boiled for 3 min before loading. A Gene Ruler 1 kb protein marker was used as a broad range protein standard to estimate the

molecular weight of the proteins (ThermoFisher Scientific, Waltham, Massachusetts, USA). The protein sample was separated at room temperature with a current of 20mA. The proteins were stained with Coomassie brilliant blue G-250 (Bio-Rad, Hercules, California, United States).

### **Western blot analysis**

Western blot analysis was performed with the purified proteins separated by SDS-PAGE were transferred onto a polyvinylidene fluoride membrane using a mini trans-blot electrophoretic transfer cell (Bio-Rad, Hercules, California, United States). To detect the His-tagged recombinant protein, a His-probe (H-3) monoclonal anti-body (Santa Cruz) and a goat anti-mouse IgG-HRP (Santa Cruz) were used as the primary and secondary antibodies, respectively.

### **Circular Dichroism studies**

Measurements of *tlyAE59Q* mutant was performed using a Chirascan CD spectrometer (Applied Photophysics). Cuvette path length used was 1 mm, and sample concentrations was 0.80 mg/ml. Protein buffer contained 10 mM sodium phosphate, pH 8.0, 100 mM NaCl. The purity of samples were checked by SDS-PAGE and size-exclusion chromatography. Each spectrum was averaged from four repeated scans ranging between 190 and 260 nm at a scan rate of 1.25 nm/s. Raw data were corrected by subtracting the contribution of the buffer to the signal.

### ***tlyA* sequence analysis**

The sequence of *Mycobacterium tuberculosis tlyA*, along with other *tlyAs* were retrieved from NCBI database (Pruitt *et al.*, (2005)). Sequence identity was verified by doing homology searches using the basic local alignment search tool (BLAST) algorithm

(Stephan *et al.*, 1990). Primary structure analysis of *tlyA* was done using the ProtParam tool. (<http://www.expasy.ch/tools/protparam.html>). The Clustal Omega multiple sequence alignment program was used to align the *tlyA* sequences (Sievers *et al.*, 2011). The ESPript server was used for generating secondary structure elements and to produce a representation of the sequence alignment (Gouet *et al.*, 1999).

### Comparative molecular modelling of *tlyA*

Homology modeling for *tlyA-Mtb* was performed in the following sequential steps: template selection from Protein Data Bank (PDB), sequence-template alignment, model building, model refinement and validation. Template search for *Mtb-tlyA* was done using NCBI-BLAST search tool against PDB database. PDB format files of the crystal structure of CTD of *Mtb-tlyA* which is available in the protein data bank (PDB ID: 5EOV) was downloaded. *Mtb-tlyA* CTD crystal structure was used as a template to generate a comparative 3D model of *Mtb-tlyA* full length by I-Tasser Server (Roy *et al.*, 2010). I-Tasser Server generated several preliminary models which were ranked based on their C-scores. Five sets of models having lowest C-scores were selected and stereochemical quality of each was assessed by PROCHECK (Laskowski *et al.*, 1993). The model with the least number of residues in the disallowed region was further refined for relieving steric clashes and improper contacts. Loop refinement tool of MODELLER was used in an iterative fashion to refine the loop conformation of the model. Structural validation after each loop refinement step was done using ERRAT plot which gives a measure of the structural error at each residue in the protein. This process was repeated iteratively until most of the amino acid residues were below 95% cut-off value in ERRAT plot (Colovos and Yeates, 1993). The

refined model was further validated by VERIFY-3D of SAVES server (<http://nihserver.mbi.ucla.edu/SAVES/>). ProSA 2003 was used to evaluate the generated 3D structure model of protein for potential errors (Wiederstein and Sippl, 2007). Model for visualization and superimposition of the model with the crystal structure of *tlyA* CTD was generated using PyMOL (DeLano 2002).

### Results and Discussion

#### Cloning and primary structure analysis

The gene search of *Mycobacterium* Genome Database Sequences for *tlyA* reveals a single copy of gene in *Mycobacterium* species. To clone E59Q mutant from *Mtb*, oligonucleotide primers were designed based on the gene sequence of *tlyA*. PCR was carried out and a ~804bp fragment was amplified from *Mtb* H37Rv chromosomal DNA. The PCR product was purified by gel extraction method, cloned into pETDuet-1 vector and sequenced. The clone thus obtained was confirmed by DNA sequencing. Such a cloning strategy resulted in the expression of *Mtb-tlyAE59Q* with 16 additional amino acids (MGSSHHHHH HSQDPNS) generated when expressed in *E.coli*.

BLASTP search showed that the primary structure of *Mtb-tlyA* has highest sequence identity with *mycobacterium canettitlyA*, which showed 99% sequence identity, whereas other *mycobacterium* species showed sequence identity of over 80%. Sequence alignment of different *tlyA* proteins were represented in Figure. 1.

Nucleotide sequence analysis of cloned *Mtb-tlyA* encodes for 268 amino acids with a predicted molecular mass of ~29.9kDa including 16 additional amino acids, molecular weight of E59Q *tlyA* is consistent

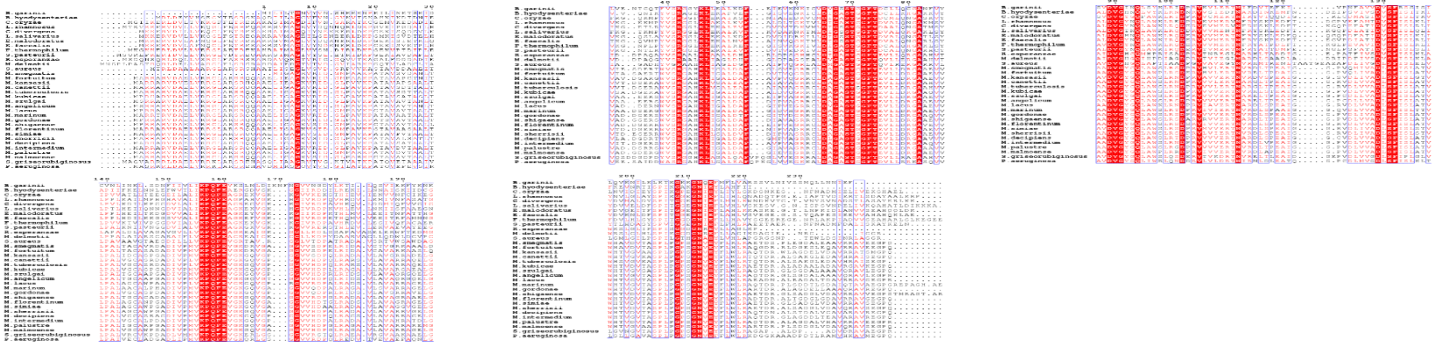
with the *tlyA* native protein published (Rahman *et al.*, 2010).

### Expression and purification

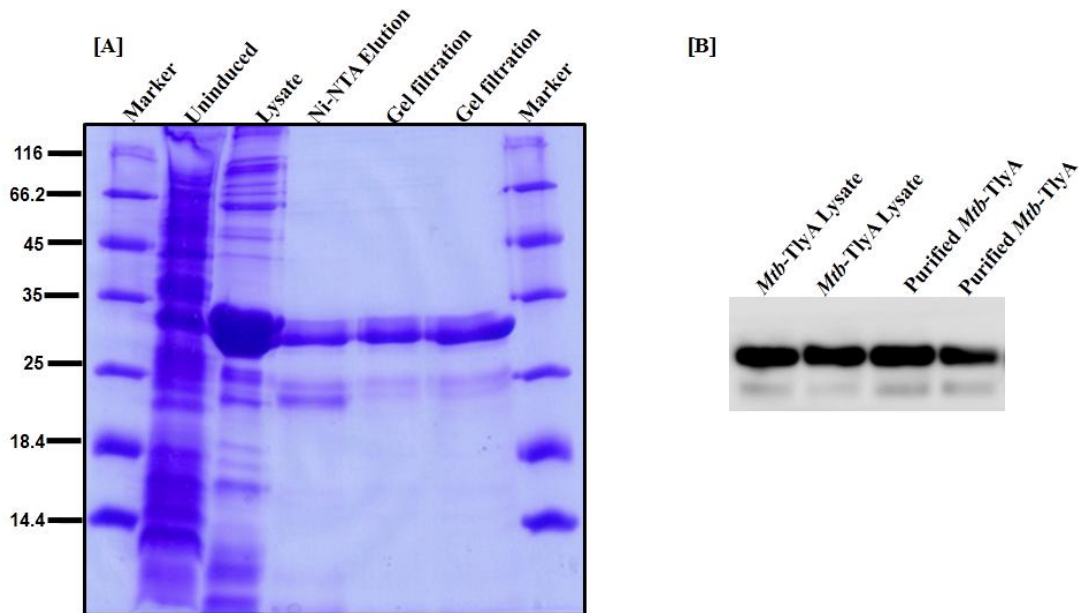
To achieve high level expression of *Mtb-tlyAE59Q*, pETDuet-1 vector system was used as the expression vector which harbours a strong promoter, T7. pETDuet-1-*Mtb-tlyA* E59Q, with 16 additional amino acids including six histidines was transformed to

*E.coli* strains BL21 (star). The recombinant protein expression level was high when overproduced. Soluble form of the protein was detected in the BL21 (star) strain, His-tagged *tlyAE59Q* was confirmed by Western blot analysis using  $\alpha$ -his antibody (Figure.2B). To identify optimum temperature for protein production, over expression of *Mtb-tlyA* from (BL-21(star)) was analyzed with different temperature ranging from 16-37° C after 12 h of induction with IPTG (Data not shown).

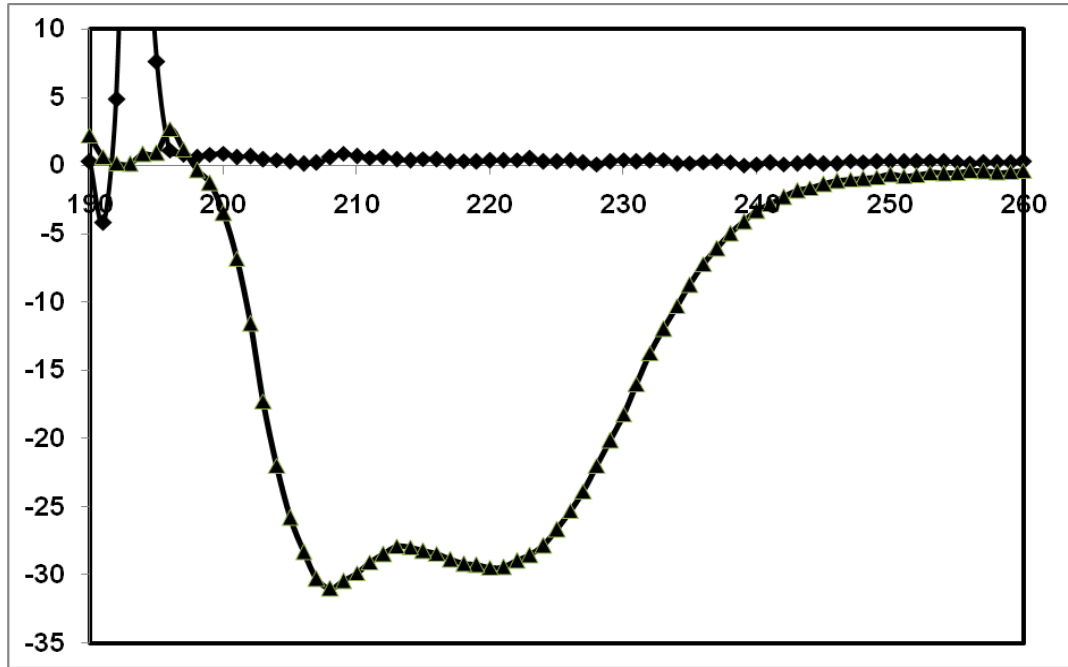
**Fig.1** Multiple sequence alignment of *tlyA* from different organisms was generated using Clustal Omega and figure was prepared using ESPrpt 3.0.



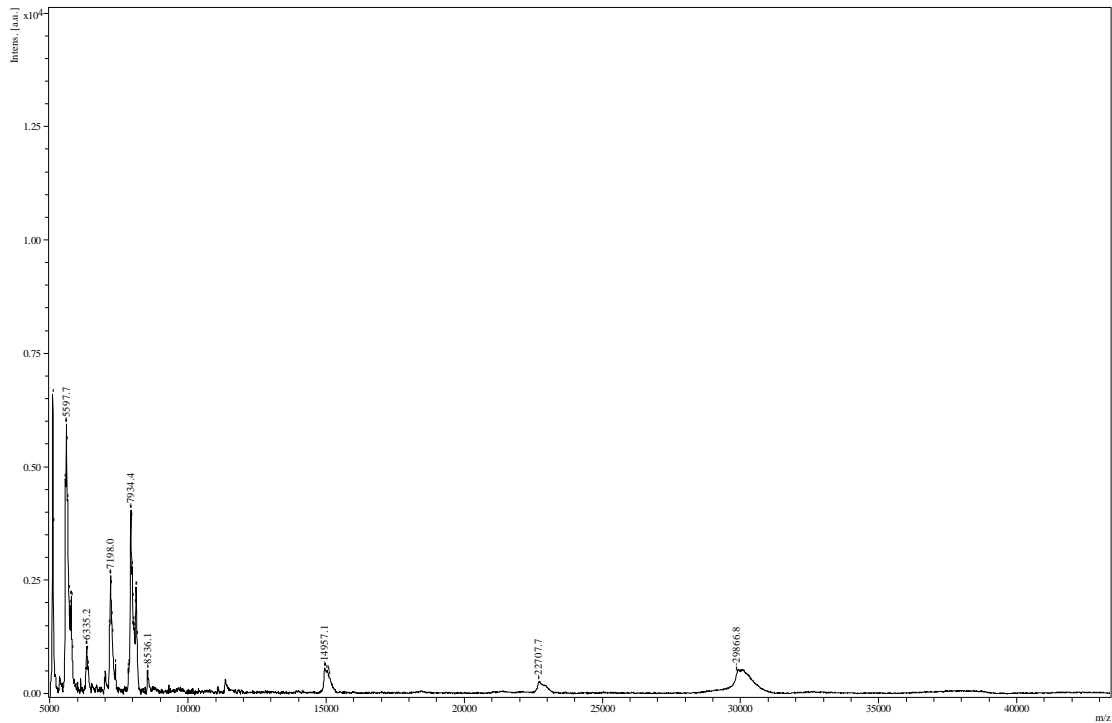
**Fig.2** (A) Purification profile of *Mtb-tlyA* E59Q. (B) Western blot identification of His-*Mtb-tlyA* E59Q by  $\alpha$ -his antibody.



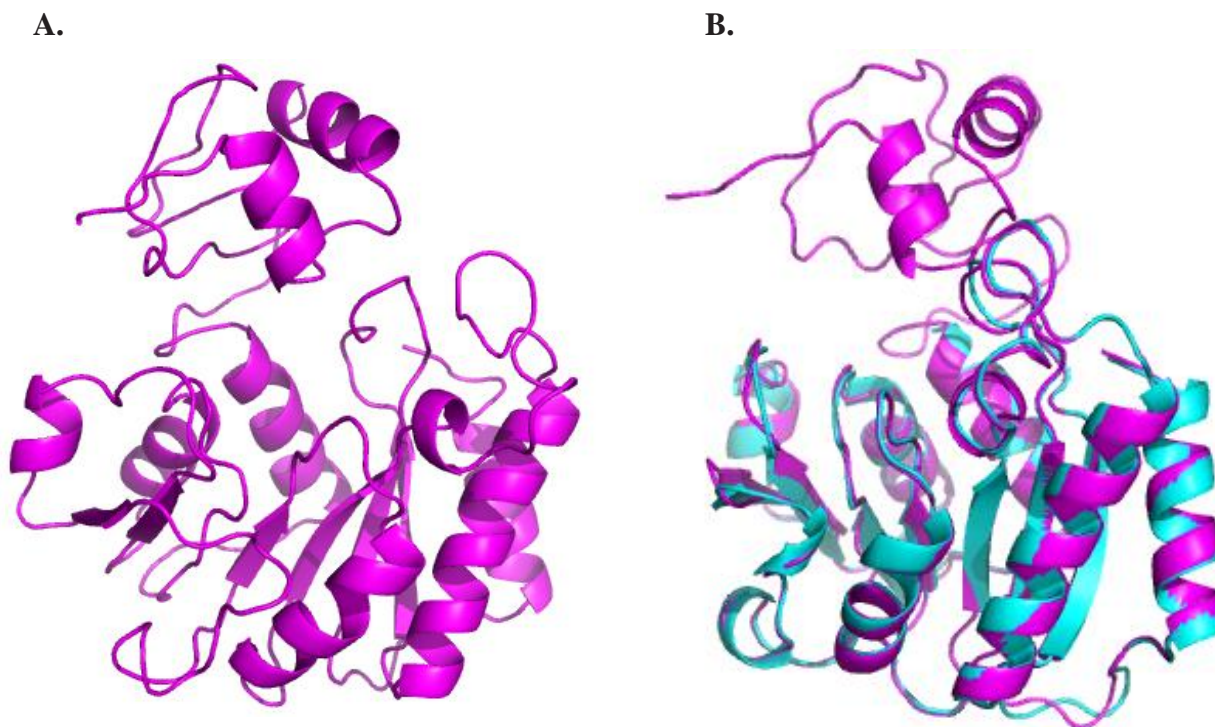
**Fig.3** CD spectroscopic analysis *Mtb*-tlyAE59Q. CD studies of *Mtb*-tlyA shows  $\alpha/\beta$  pattern of structured protein.



**Fig.4** Mass spectrum of *Mtb*-tlyA E59Q obtained by MALDI-TOF.



**Fig.5** (A) *Mtb-tlyA* generated model by I-Tasser Server (B) Superposition of *Mtb-TlyA* model over crystal structure of *Mtb-TlyA* CTD.



His-*tlyA*E59Q at the N-terminus which facilitated purification by Ni-NTA affinity chromatography. Gel filtration chromatography was performed after Ni<sup>2+</sup> affinity chromatography, where we could purify *tlyA*E59Q to homogeneity. The native protein reported to have autocatalytic activity (Witek *et al.*, 2017), whereas E59Q did not show any autocatalytic activity. Gel filtration profile showed single predominant peak indicating the *Mtb-tlyA*E59Q is homogenous and which was further confirmed by SDS-PAGE (Figure.2A). Fractions corresponding to protein on SDS-PAGE were pooled, concentrated and stored in -80°C.

The CD spectrum of His-*tlyA* native and E59Q showed the pattern of well folded protein with a mixed  $\alpha/\beta$  structure and protein was quite stable even after storage in room temperature (Figure.3) (Whitmore *et al.*,

2008). Mass spectrometry analysis indicated the protein is ~30kDa which is evident from the previous reports (Witek *et al.*, 2017) (Figure.4). Previous studies indicated that Glu<sup>59</sup> surface exposed amino acid separates between NTD and CTD of *Mtb-tlyA*. Studies indicated that NTD and CTD were intact even after cleavage under the solution conditions (Witek *et al.*, 2017).

#### **Molecular Model of *tlyA* full length protein**

Previous structural studies of *Mtb-tlyA* CTD showed RrmJ/FtsJ Rossmann-like methyltransferase fold and also overlays well with other Class I methyltransferases (Witek *et al.*, 2017).

Studies also indicated that methyltransferase fold of *tlyA* is not sufficient for SAM binding, and thus, one or more amino acids of the



aminoterminal residues of *tlyA* play an important role in SAM binding (Witek *et al.*, 2017). Since *Mtb-tlyA* NTD plays an important role and is dispensable for interaction with the substrate, *Mtb-tlyA* N-terminus structure plays an important role.

*Mtb-tlyA* full length model was generated using crystallographic 3D structure of *Mtb-tlyA* CTD (PDB ID:5EOV) as template (Figure.5). The generated model was subjected to refinement, loop modeling and energy minimization. PROCHECK, Verify-3D and ERRAT plot were used for determining the stereo-chemical parameters of the energy minimized model of *Mtb-tlyA*.

Ramachandran plot of the 3D model generated by PROCHECK shows 90% in allowed region, 7% in generously allowed region and 3% in disallowed region. ERRAT plot gives an overall quality factor of 94.966 to the modeled structure. ProSA(2003) analysis showed that protein folding energy of our modeled structure is in good agreement with that of the template.

### Acknowledgement

One of the author Shivaleela acknowledge TEQIP-III to Basavehwar Engineering College (A), Bagalkot for the financial support.

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**How to cite this article:**

Shivaleela, V. B., ShaikKalimulla Niazi, P. Sharada and Giri Gowda, K. 2019. *Mycobacterium tuberculosis* tlyA E59Q is Stable Compared to the Native Protein. *Int.J.Curr.Microbiol.App.Sci.* 8(09): 2203-2212. doi: <https://doi.org/10.20546/ijcmas.2019.809.254>