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Cloning, expression, and refolding of N-succinyl-l-l diaminopimelic acid desuccinylase (DapE) of Mycobacterium tuberculosis

Chinmay P. Umbarkar[1], Prashant S. Thakre[2], Supriya A. Kashikar[3], Niraj A. Ghanwate[4], Vinod B. Agarkar[5]*

[1,2,3,5] GeNext Genomics Pvt. Ltd., 103 Abhyankar Nagar, Nagpur 440010, MS, India

[1, 4] Department of Microbiology, Sant Gadge Baba Amravati University, Amravati 444602, MS, India

*Corresponding author (*e-mail*): vinodagarkar@gng.asia

Abstract: **Mycobacterium tuberculosis (Mtb) infects nearly ten million individuals worldwide every year and problem is even worst due to increase in number of multi drug resistance. Tuberculosis elimination program under National Strategic Plan need progressive studies on protein, enzyme and pathways of Mtb. This would facilitate the urgent need to design and development of additional novel/potent antitubercular agents. The Lysine/DAP biosynthetic pathway is a promising target as it finds specific role in cell wall and amino acid biosynthesis. In this study, we predominantly aimed an enzyme DapE from M. tuberculosis H37Rv (Mtb-DapE) consists of 354 amino acids with a theoretical molecular weight of 37,240 Daltons. Mtb-DapE gene was amplified and cloned into bacterial expression vectors. The recombinant DapE protein was successfully purified from inclusion bodies with a high yield. This structurally compatible well folded protein as assessed by the CD spectral analyses could thus be useful for advanced anti-tubercular targeting studies.**

Keywords: **Mycobacterium tuberculosis, Lysine/DAP biosynthetic pathway, DapE, Protein.**

1. INTRODUCTION

Tuberculosis (TB) is an ancient communicable airborne and the second most common infectious disease from single infectious agent i.e. *Mycobacterium tuberculosis (Mtb). Mtb* is the cause of highest mortality after HIV. In 2017, there were 12 million cases of TB out of which 14 million were fatal (WHO Global tuberculosis report 2018). In addition the emergence of multidrug resistant (MDR) and extensively drug-resistant (XDR) strains has caused the increase in TB cases. The global research community is concerned about a formidable increase of *Mtb* burden specifically in developing countries. In 1992, India together with World Health Organization (WHO) and Swedish International Development Agency (SIDA) initiated the Revised National Tuberculosis Control Program (RNTCP). The campaign against TB continued and as a next step the government constituted the National Strategic Plan (NSP) for TB Elimination 2017-2025 (https://tbcindiagovin/) under the TB-free India campaign.

According to Infectious Disease Society of America, the control of infectious diseases will require several new systemic antibacterial drugs. The developments of new drug molecules are essential to inhibit the metabolic pathways of *Mtb* Lysine and meso-diaminopimelate biosynthesis. Dap pathway is indispensable for protein and peptidoglycan cell wall synthesis of *Mtb* (Girodeau JM et al. 1986, Hutton CA et al 2007). The interference in catalysis of responsible enzymes causes instability of peptidoglycan and substantially cell death (Cirillo JD et al. 1994, Born TL et al. 1998, Wheeler PR et al. 2005). Meso-DAP biosynthesis exists only in bacteria and is absent in mammals (Cox RJ et al. 2000, Diaper CM et al. 2005, Hudson AO et al. 2005). DapE (N-succinyl-l-l diaminopimelic acid desuccinylase) protein of *Mtb* does not share significant identity with other bacterial species DapE proteins (Nocek B et al. 2014). The significance and studies of

DapE pathway and responsible enzymes augmented with the anticipation for new drug targets (Usha V et al. 2012, Usha V et al. 2016, Fakhar J et al. 2016).

Protein expression, purification, high and quality yield are primary steps for the basis of numerous biochemical and biomedical studies. Here, our study provides a high-yielding refolded *Mtb*-DapE protein that could be useful in future studies aimed at developing novel therapeutics against *Mtb* (MDR and/or XDR).

2. MATERIALS AND METHODS

The reagents used in this study: Restriction enzymes (New England Biolabs), Protein expression plasmids and *Ecoli* strains (Novagen), Ni-NTA (Qiagen), IPTG (MP Biomedical), LB media (Amresco), Antibiotics (Hi-media) Buffers and other chemicals (SIGMA and Hi-Media), Western Blot NC Paper (Pall Life Sciences), 6X-His monoclonal antibody (Pierce).

Genomic DNA isolation

Genomic DNA isolated and purified using *Mycobacterium Tuberculosis* Protocols (Helden PD et al. 2001). In brief, *M. tuberculosis H37Rv* culture sealed tube heated at 80°C for 1 hour. Class III biosafety cabinet was used for added safety steps. Extraction buffer containing 50 mM Tris-HCl, 25 mM EDTA, 5% mono-sodium glutamate, pH 7.4 along with 5 mm 30 glass beads added in bacterial culture and 2–3 min full speed vortex used to disrupt bacterial colonies. 400 µL of 50 mg/mL lysozyme and 10 µL of 10 mg/mL RNAase was added and incubated 2 hour at 37°C for cell lysis and RNA degradation. Proteinase K was used for removal of protein contamination. Further purification was performed using phenol, chloroform and isoamyl alcohol (25:24:1) DNA extraction method. Finally alcohol precipitated dried *Mtb* genomic DNA (g*Mtb*) suspended in lukewarm TE buffer and stored at – 80°C.

Construction of recombinant plasmid

Gene specific forward 5'-ATGCTGGATTTGCGCGGGGACC-3' and reverse 5'-CTAGCCACCCAGGTATC GGCGCAG-3' primers designed after retrieving gene sequence (DapE Gene ID: 887386) from NCBI nucleotide database. Full DapE (1065 bp) gene amplified by PCR from gMtb. Second PCR performed using Nde 1 and Xho 1 restriction sites inserted in the forward 5'-ATTTACATATGCTGGATTTGCGCGGGGACCC-3' and reverse 5'-ATACTCGAG ACCCAGGTATCGGCGCAGCA-3' primers respectively. Amplified final DapE gene product and pET vectors were digested by *Nde 1* and *Xho 1* enzymes, analyzed and purified on 1% agarose gel electrophoresis. The resulted DapE gene ligated in pET 21a and 28a vector and transformed in chemically competent *EColi* DH5α. The positive clones screened by gene specific primers and restriction digestions. The final DapE C-terminus six histidine tag pET21a recombinant plasmid named as DapEHM2. However with additional in built N-terminus six histidine including 20 amino acids recombinant pET28a plasmid named as DapEHM28. The forward and reverse sequencing results confirmed the correct orientation and sequence.

Protein expression

DapEHM21 and DapEHM28 plasmids transformed in BL21 (DE3) competent cells. DapEHM21\BL21 and DapEHM28\BL21 cells inoculated in LB growth media supplemented with ampicillin (100 ug/ml) and kanamycin (50 ug/ml) respectively. Overnight primary culture re-inoculated in antibiotic selection LB media shaking flasks and allowed it to grow for 3 hours at 37°C at 220 rpm. Half of the culture transferred at 18°C and after 30 minutes both (18°C and 37°C) cultures induced by 0.1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). The 37°C culture harvested after next 3-4 hours. However 18°C culture harvested after 18-20 hours. Harvested cells proceeded for sonication on ice in cold extraction buffer 50 mM Tris-HCl pH 8.0, 300 mM NaCl, 10% glycerol, 10 mM βME and 1mM PMSF. The supernatant was collected after 30 minutes centrifugation at 16000 rpm and stored at -80°C .

Dissolution of inclusion bodies

The remaining cell pellet (insoluble inclusion body form) of 37°C culture was re-suspended in extraction buffer containing 01% Triton X-100. Shaking at room temperature for 30-40 minutes and centrifuged at 16000 rpm for 20 minutes. Obtained pellet was washed 2-3 times with 50 mM Tris-HCl pH 8.0 and 500 mM NaCl buffer. Finally, inclusion bodies separated from suspension by centrifugation at 16000 rpm. DapE inclusion bodies suspended by mixing with pipetting in buffer A containing 50 mM Tris-HCl pH 8.0, 300 mM NaCl, 20 % glycerol, 10 mM βME and 8 M Urea.

Protein purification

The temperature 4°C was maintained during all protein purification steps. The solubilized cell pellet comprising inclusion bodies centrifuged at 18000 rpm for 60 minutes to remove insoluble particulates. The clarified supernatant was allowed to bind Ni-NTA resin pre-equilibrated with buffer A for overnight with continuous rotation. Protein bound resin packed in column and washed once with buffer A. Two more 0.1 ml/minute flow rate washes were applied using decreasing concentration of 6M, 4 M and 2 M urea containing buffer A. The column was extensively washed with buffer B containing 50 mM Tris-HCl pH 8.0, 300 mM NaCl, 20 % glycerol, 10 mM βME, 2M Urea and 10 mM Imidazole. The bound protein was eluted with buffer B containing 200 mM Imidazole. Eluted fractions were subjected for overnight dialysis in buffer C containing 30 mMTrisHCl pH 8.0, 112 mM NaCl, 1mM DTT, 1mM EDTA and 10 % glycerol. The refolded DapE centrifuged at 18000 rpm for 1 hour. Collected protein solution subjected to increase NaCl concentration up to 150 mM and filtered through 0.22 µm membrane filter. Subsequently, as final polishing step DapE was loaded on to size exclusion chromatography column (Superdex S200 16/60 GE Healthcare) attached with AKTA purifier 100. The equilibration and elution buffer D containing 30 mM Tris HCl pH 8.0, 300 mM NaCl, 2% Glycerol and 1 mM DTT was used. Protein purity and molecular weight was analyzed on 12% SDS-PAGE protein estimation performed by Bradford assay (Sigma).

Western blot analysis of 6X-Histidine DapE

Western blotting was performed on 0.45 µm nitro cellulose membrane by standard procedures (Towbin H et al. 1979). Briefly, 6X-His tag DapE protein electrophoretically transferred on membrane after SDS-PAGE. The blocking performed with 2% BSA in Tris buffer saline overnight at 4°C. Membrane was washed thrice with 1X TBS supplemented with 0.05% Tween- 20. Blot incubated with mouse 6X anti His antibody used as per manufacturer instructions. The blot was developed using TMB as substrate. DapE was successfully detected with anti His antibody at very low concentration up to 62 ng.

Secondary structure analysis (CD spectroscopy)

The circular dichroism measurement of refolded DapEHM21 was estimated using JASCO J-715 CD spectrometer (Jasco J-715 CD Operating Instructions Manual). The lyophilized protein suspension concentration adjusted at the 0.1 mg/ml in sterile milli Q water. The CD spectra were measured over the wavelength of 190-260 nm, band width of 1 nm, scanning speed of 500 nm/min and temperature at 25 °C.

3. RESULTS AND DISCUSSION

The quality and quantity of genomic DNA of *M. tuberculosis H37Rv* was verified on 0.8% agarose gel electrophoresis (Fig. 1A). A 1062 bp DapE gene product amplified by PCR (Fig. 1B) and cloned into pET21 and pET28 vector yielding DapEHM21 and DapEHM28 expression plasmids (Fig. 1C). The correct orientation and sequence of cloned genes were verified and confirmed following the nucleotide sequencing analysis at Bioserve Biotechnologies India (data not shown). However, DapEHM21 construct was built with extra C-terminus six histidine-tag (6XHis) while DapEHM28 with pET28a vector in-built N-terminus 20 amino acids. The concluded plasmids were transformed in BL21 (DE3) cells and grown on large scale for expression of DapE protein. An anticipated expression level or purity was not significantly affected by extended inbuilt 20 amino acids protein sequence including extra six histidine tag at N-terminal of pET28 vector as compared to without N-terminal His tag of DapEHM21\BL21 construct. DapE cloned and expressed using pET21 vector was initially tried for native protein expression and purification. The reduction of protein induction temperature and different IPTG concentrations were performed for native protein expression. Controlling aggregation tendency of DapE was a challenge. Visual inspection, centrifugation and Bradford protein assay was performed in monitoring of native form of protein expression and aggregation (Mahler HC et al. 2009). Aggregation to degradation problem continued albeit storage at 4 °C or -20 °C, changing pH and buffer constituents. The storage in 20% glycerol containing Tris-HCl buffer pH 8.0, 300 mM NaCl and 1 mM DTT improved the stability only by another 15-20 days. However, yield around 20-50% decreased in purified protein concentrations. Unexpectedly only 0.2-0.5 mg native form of protein was purified from 1 L culture. The expression of DapE controlled by 0.1 mM IPTG at 37 °C formed the inclusion bodies (Fig. 2). The suspension of inclusion bodies in 0.1 % Triton X-100 containing extraction buffer facilitated for bodies cleaning process. Dissolution in high molarity urea containing buffer, glycerol and BME helped for complete denaturation of protein. The protein bound Ni-NTA resin washed with decreasing molarity of urea with slow flow rate helped for in-column refolding of the protein. Finally decreasing 2 to zero molarity of urea concentration by dialysis helped for yielding well folded protein. AKTA attached size exclusion chromatography column was used in final protein purification finishing process. By SDS-PAGE analysis more than 96% protein purity was found. The total of 15-20 mg of protein was purified from one liter culture (Fig. 2 C). Purified protein was analyzed by western blot using anti 6XHis tag antibody to confirm intact six histidine

(Fig. 3 A). The CD spectral data analysis of refolded *Mtb*-DapE showed well folded protein in solution (Fig. 3 B). Beta structure selection server estimated 29.4 % helical structure using CD spectral data (Micsonai A et al. 2015). However, alpha amylase was the closest secondary structure predicted by CAPITO (Wiedemann C et al. 2013). The *Mtb*-DapE protein sequence alignment with DapE from *Corynebacterium glutamicum* ATCC 13032 showed 55 % identity (Fig. 4) (https://blastncbinlmnihgov/). However other DapE from "ESKAPE" pathogens (Gillner DM et al. 2009, Nocek B et al. 2014) were around 20-24 % identical. ArgE crystal structure (PDB ID: 3tx8) plausible molecular replacement candidate for the solution of DapE protein crystal structure determination contains 29.8 % helical secondary structure. Thus the present refolded high yield *Mtb*-DapE protein could be used for further investigations.

4. CONCLUSION

The sequence alignment of DapE (N-succinyl-l-l diaminopimelic acid desuccinylase) of *Mycobacterium tuberculosis* shows fewer identities with other prokaryotes or eukaryotes enzymes (Gillner DM et al. 2009). The comprehensive study of DapE is necessary to understand this potential drug target against tuberculosis. Linda Reinhard et al. (2012) cloned, expressed, purified and crystallized the DapE protein in native form though crystal structure not offered. Here, we expressed DapE protein as inclusion bodies and purified to expand around 30 times more protein. However the refolded purified DapE protein constituted in 50 mM Tris-HCl buffer pH 8.0, 300 mM NaCl, 1 mM DTT and 2% glycerol stored at -80 °C in aliquots for more than 8 months without aggregation problems. The present study would be indispensable for further research where the large quantity of *Mtb*-DapE protein is required.

Author's contributions: CU, PT and VA performed the experiments. VA conceived and designed the experiments. CU, SK and VA wrote the manuscript. CU, SK, NG and VA contributed in data analysis. All authors read and approved the manuscript.

Conflict of interest: There is no conflict of interest for the authors.

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APPENDICES - A

FIGURES and LEGENDS

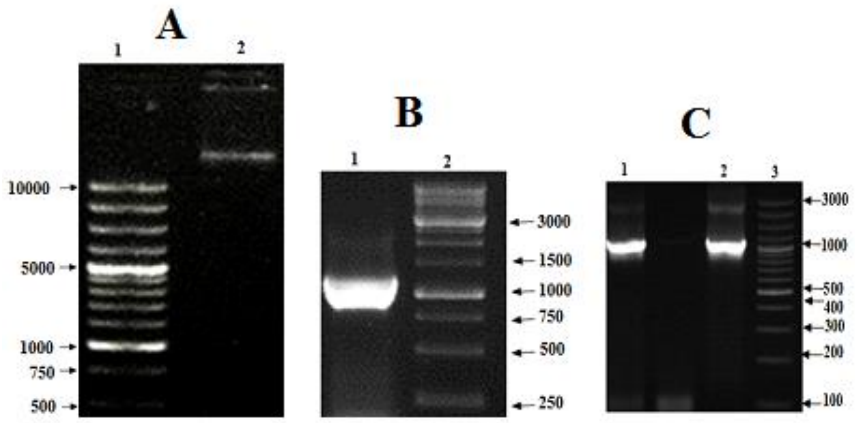

Figure 1: 0.8-1 % Agarose gel (A) 1. DNA ladder, 2. *M. tuberculosis H37Rv* genomic DNA; **(B) 1.** DapE gene amplified by gene specific primers, **2.** DNA ladder; **(C)** ***Construct clone verification by colony PCR*** **1.** DapEHM21, **2.** DapEHM28

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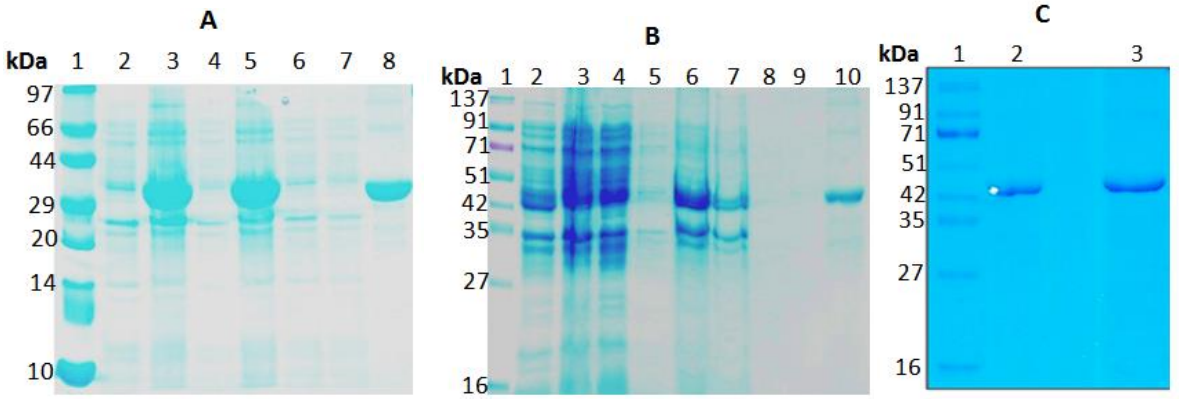

Figure 2: DapE protein expression and purification by Ni-NTA chromatography (A) ***DapEHM21 protein*** **1.** Protein MW marker, **2.** Uninduced, **3.** Induced whole cells, **4.** Supernatant, **5.** Inclusion bodies in urea, **6.** Flow through, **7.** Wash, **8.** Elution. **(B)** ***DapEHM28 protein*** **1.** Protein MW marker, **2.** Uninduced, **3.** Induced supernatant, **4.** Induced whole cells, **5.** Supernatant, **6.** Inclusion bodies in urea, **7.** Flow through, **8.** Wash 1, **9.** Wash 2, **10.** Elution **(C)** ***Final purification by size exclusion chromatography*** **1.** Protein MW marker, **2.** DapEHM21 protein, **3.** DapEHM28 protein

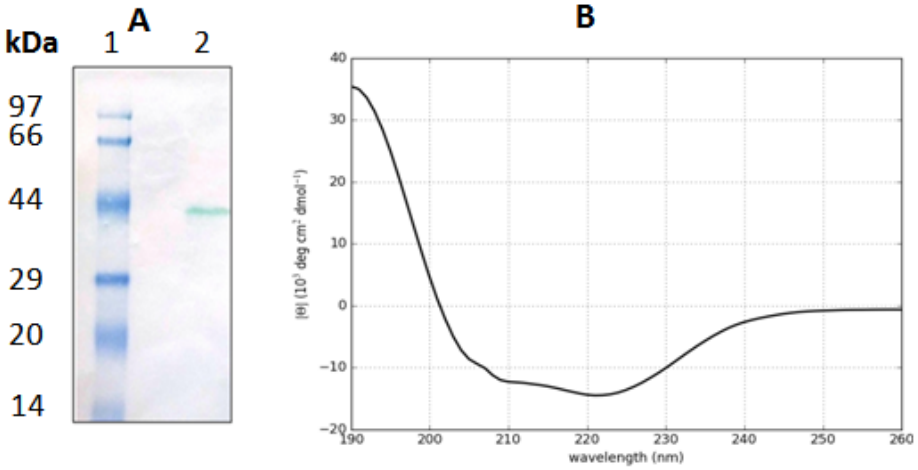

Figure 3: (A) Western blot analysis of 6X-Histidine DapEHM21 protein (**B)** CD spectra of DapEHM21 protein

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DapE (C.glutamicum)   MNSELKPGLDLLGDPIVLTQRLVDIPSPSGQEKQIADEIEDALRNLNLPGVEVFRF----NNN-VLART--NRGLASRVM
DapE (M.tuberculosis) -------MLDLRGDPIELTAALIDIPSESRKEARIADEVEAALRA-QASGFEIIR-----NGNAVLARTKLNR--SSRVL

DapE (C.glutamicum)   LAGHIDTVPIAD-------NLPSRVEDGIMYGCGTVDMKSGLAVYLHTFATLATS-TELKHDLTLIAYECEEVADHLNGL
DapE (M.tuberculosis) LAGHLDTVPVAG-------NLPSRRENDQLHGCGAADMKSGDAVFLHLAATLAEP-T---HDLTLVFYDCEEIDSAANGL

DapE (C.glutamicum)   GHIRDEHPEWLAADLALLGEPTGG-----WIEAGCQGNLRIKVTAHGVRAHSAR-----SWLGDNAMHKLSPIISKVAAY
DapE (M.tuberculosis) GRIQRELPDWLSADVAILGEPTAG-----CIEAGCQGTLRVVLSVTGTRAHSAR-----SWLGDNAIHKLGAVLDRLAVY

DapE (C.glutamicum)   QAAEVNIDGLTYREGLNIVFCESGV-----ANNVIPDLAWMNLNFRFAPNRDLNEAIEHVVETLELDGQDGI--EWAVED
DapE (M.tuberculosis) RARSVDIDGCTYREGLSAVRVAGGV-----AGNVIPDAASVTINYRFAPDRSVAAALQHVHDVF--DGLD-VQIEQ--TD

DapE (C.glutamicum)   GAGGALPGLGQQVTSGLIDAV-----GREKIRAKFGWTDVSRFSAMGIPALNFGAGDPSFAHKRDEQCPVEQITDVAAI-
DapE (M.tuberculosis) AAAGALPGLSEPAAKALVEAA-----GGQ-VRAKYGWTDVSRFAALGIPAVNYGPGDPNLAHCRDERVPVGNIT--AAVD

DapE (C.glutamicum)   -LKQYLSE----
DapE (M.tuberculosis) LLRRYLGG----
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Figure 4: Amino acids sequence alignment of Mtb-DapE with DapE from *Corynebacterium glutamicum* ATCC 13032. Bold underlined residues involved in metal binding.