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Cloning, expression, purification and preliminary oligomerisation analysis of recombinant protein *Mycobacterium tuberculosis* Rv1288

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ABSTRACT

Aims: LysM containing-protein is widely distributed in all domains of life and this kind of protein is essential for various biological activities in living organisms. Rv1288, a LysM containing-protein with esterase, was found in *Mycobacterium tuberculosis*. Biophysical studies revealed that the protein is responsible for modulating lipid metabolism that enables pathogens to survive under extreme conditions and decrease the permeability of the pathogen's cell wall to drug therapeutic agents. However, recognition and interaction between the protein, lipid and carbohydrate moieties at the molecular level remains largely unknown and must be investigated. Therefore, a production of recombinant protein Rv1288 should be performed to aid the study.
Methodology and results: In this study, we cloned the full-length cDNA of Rv1288 from *M. tuberculosis* strain H37Rv and expressed it in pET-24d- *Escherichia coli* BL21(*DE3*) cells. Affinity and size exclusion chromatography methods purified the protein, and its preliminary oligomerisation state was determined based on a calculated apparent molecular weight of the protein. Rv1288 was expressed as a soluble protein at 20 °C, induced with 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG). The calculated apparent molecular weight suggested that the Rv1288 protein formed a hexamer in solution.
Conclusion, significance and impact of study: All the methods involved in this study to produce the recombinant Rv1288-pET24d and its soluble protein in *E. coli* cells have been described. Hence, it can be implemented for future studies.

Keywords: Cloning, expression, LysM, oligomerisation, purification

INTRODUCTION

LysM domain-containing protein is initially found in a lysozyme-like enzyme from *Bacillus* phage phi 29 (Garvey *et al.*, 1986). Studies have revealed that the protein contains two different modules on a single polypeptide, LysM and catalytic domains (Ruhland *et al.*, 1993; Bateman and Bycroft, 2000; Visweswaran *et al.*, 2014; Kitaoku *et al.*, 2017). The LysM domain is a module of approximately 50 residues (Desvaux *et al.*, 2006) that recognizes N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), major constituents of peptidoglycan and chitin molecules including lipochito-oligosaccharides (Béliveau *et al.*, 1991; Steen *et al.*, 2008; Oguiza, 2022). While the catalytic domain harbours enzymatic functions responsible for oligosaccharide and lipid management, including glycosidases, amidases, esterases, lysozymes and glycosaminidases (Vollmer *et al.*, 2008). These multi-module proteins are widely distributed in all domains of life (Radutoiu *et al.*, 2003; Steen *et al.*, 2003).

LysM containing-proteins are regulators of various biological activities in living organisms involving cell growth, pathogenicity, virulence factors, stress response and immune evasion mechanisms (Bolton *et al.*, 2008; Liu *et al.*, 2012, Dubey *et al.*, 2020; Sánchez-Vallet *et al.*, 2020; Shumayla *et al.*, 2022). The LysM domain mediates direct binding to peptidoglycan or chitin of bacterial cells or fungi, respectively, to facilitate interaction between the catalytic domains of the associated enzymes to its substrates (Takayama *et al.*, 2005; Visweswaran *et al.*, 2014). In the example of *Staphylococcus* protein A (SpA), its LysM and SPA domains were reported cooperatively bound to peptidoglycan and IGg-Fc fragments, respectively, to avoid the pathogen from host phagocytosis (Uhlén *et al.*, 1984). In plants, LysM domain of *Ecp6*, a cell effector of *Cladosporium fulvum*, a tomato leaf pathogen, was suggested to be responsible for

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triggering signals to its catalytic neighbour which allows the molecule to hinder chitin fragments from the host immune response during cell invasion (Sánchez-Vallet *et al.*, 2020; Hu *et al.*, 2021).

Rv1288 has been suggested as one of the lysM containing-protein with carboxyl-esterase activities found in *M. tuberculosis* that is responsible as a modular for cell wall lipid metabolism, inducer for nutrient stress and involves in drug resistance of the species (Maan *et al.*, 2018). It was reported that when Rv1288 was expressed in surrogate *M. smegmatis* cells, it increased the amount of lipids production in the species, which eventually enhanced bacterial intracellular survival and decreased drug penetration into the cells. Based on sequence alignment analysis, the Rv1288 protein shared about 30% amino acid sequence identity with the Antigen 85 complex (FbpA, FbpB and FbpC) (Takayama *et al.*, 2005; Maan *et al.*, 2018). This protein plays a vital role in the pathogenicity of *M. tuberculosis*, enabling mycobacteria to evade host immune response by preventing the formation of phagolysosomes to eradicate infections caused by the pathogen (Babaki *et al.*, 2017). Other Rv1288 homologs have also been found in various clinically important *Mycobacterium* species, including *M. bovis*, *M. avium* and *M. marinum*. This shows that Rv1288 is vital to *Mycobacterium* species and is possibly responsible for its pathogenesis, dormancy and drug resistance mechanism (Quinonez *et al.*, 2022). This protein can potentially be a good candidate for new anti-TB drug targets.

Biophysical properties of the Rv1288 protein have been explored under different stress conditions to understand its functions in phenotypic variation in *Mycobacterium* species. However, a deeper understanding of the recognition and interaction between the protein, carbohydrate and lipid moieties at a molecular level remains largely unknown and needs to be investigated. The information will shed light on the mechanism of the novel *M. tuberculosis* inhibitors and drug therapeutics design and development. Therefore, this study was conducted to produce recombinant *M. tuberculosis* Rv1288 in *E. coli* as an initial step to aid the three-dimensional structure and functional studies of the protein with its substrates.

MATERIALS AND METHODS

Homologs search

Proteins containing multiple tandem repeats of the LysM domain were identified in the NCBI GenBank by a BLASTp search using the conserved LysM motif GDTLxxI. Such proteins were found in various organisms, including *Mycobacteria*, *Acinetobacter*, *Rhodococcus* and *Nocardia*. Analysis of the protein sequence of *M. tuberculosis* Rv1288, a protein identified in the genome sequence of *M. tuberculosis* strain H37Rv in the UniProt database (Protein ID: P9WM39), indicated it consisted of three tandem repeats of the LysM domain with a hypothetical esterase.

Amplification of *Rv1288* gene by PCR assay

Mycobacterium tuberculosis Rv1288 gene was amplified from genomic DNA of *M. tuberculosis* strain H37Rv, which was obtained from the Institute for Respiratory Medicine (IPR), Kuala Lumpur, by polymerase chain reaction utilising a pair of primers (Forward primer: 5'-CCATGGTCAGCACACATGCGGTTGT-3'; Reverse primer: 5'-CTCGAGTTCGGCGCGTTCTGCGGCC-3') containing restriction enzyme sequences of *Nco*I and *Xho*I in the forward and reverse oligonucleotides, respectively, to give a final size of amplicons ~1400 bp. All the PCR samples contained 1U of Taq DNA polymerase, 200 µmol of dNTPs, 1× PCR buffer, 10 pmol of primers and 100 ng of DNA template. PCR conditions were optimised primarily focusing on an annealing temperature. The Rv1288 gene was amplified using 3 PCR steps: 2 min at 98 °C, followed by 35 cycles of 98 °C for 35 sec, 71 °C for 35 sec and 72 °C for 60 sec, and a final extension at 72 °C for 5 min. The end PCR products were subjected to 1% agarose gel electrophoresis and the results were visualised under UV light (~365 nm).

Rv1288 DNA gene cloning

The PCR product of *Rv1288* gene and *pET24d* cloning vector (Novagen, GERMANY) were digested by using *Nco*I and *Xho*I restriction enzymes to provide sticky ends for the gene insert and the vector. The digested *Rv1288* gene and *pET24d* products were cleaned up from contaminants using a gel extraction kit (Qiagen, GERMANY). The product was ligated into a *pET24d* expression vector using ligase enzyme at 4 °C overnight. The recombinant plasmid containing the respective gene construct was propagated in *E. coli* DH5α (NEB, USA), verified by nucleotide sequencing (Illumina, USA) and then transfected into *E. coli* BL21(*DE3*) cells (NEB, USA) by heat shock transformation. The transformants were initially inoculated into SOC media and incubated for 30 min at 37 °C before plating onto LB media supplemented with 50 µg/mL kanamycin. The transformants that grew on the LB media were randomly selected for a colony PCR assay to detect the presence of the inserted gene. The universal T7 primers were utilised to amplify the *Rv1288* gene as the vector *pET24d* used a T7 promoter as a gene transcription regulator in *E. coli*. All the colonies that were positive for T7 primers were sent for DNA sequencing to analyse the inserted gene sequences and their orientation in the plasmid.

Rv1288 protein expression

Small and large-scale protein expressions were carried out on the recombinant plasmids containing the *Rv1288* gene in BL21(*DE3*) cells. The small-scale expression was initially carried out in 50 mL Luria Bertani (LB) broth containing 50 µg/mL kanamycin. The cells were induced when their optical density (OD) reached 0.6 at 600 nm with a range of isopropyl β-D-1-thiogalactopyranoside (IPTG) concentration from 0.1 mM

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to 1 mM and the grown cells were further incubated at different temperature setting to obtain the optimum conditions for Rv1288 to be expressed as a soluble protein in *E. coli* cells. Following the small-trial experiments, large-scale expression of Rv1288 was carried out using the optimal condition of 20 °C for 48 h at 110 g. The 2 L conical flask containing 500 mL of LB media and 50 mL of fresh cell cultures was grown under this condition and the cells were induced with 1 mM IPTG. The size of Rv1288 protein was expected to be ~50.7 kDa. The amount of expressed protein was determined using a Bradford protein assay at 595 nm with Coomassie Brilliant Blue dye (BioRad, USA).

Rv1288 protein purification

Prior to protein purification, the cells were harvested by centrifugation at 8,500× *g* for 20 min at 4 °C. The supernatant was discarded, and the cell pellet was sonicated in Buffer A containing NaCl (0.5 M) and Tris-HCl (1 M) (pH 8.0). The cell paste was disrupted by three bursts of sonication on ice, each for 20 sec. The soluble Rv1288 protein in the supernatant was separated from the insoluble fraction by centrifugation at 45,000× *g* at 4 °C for 15 min. The proteins were purified and eluted using an automated AKTA pure chromatography (GE Healthcare, SWEDEN) under two purification steps, affinity chromatography and size exclusion chromatography. The Rv1288 protein was applied to a 5 mL HisTrap HP nickel affinity column (GE Healthcare, SWEDEN). It was eluted under a linear gradient of 0-70% of 1 M imidazole in Buffer B with 5 times column volumes. The Rv1288 protein fractions were subjected to the 12% SDS-PAGE gel to check the purity of the protein. The protein was further purified, fractionated and eluted in Buffer A using a Superdex-200 pg (1.6 × 60 cm HiLoad) column (GE Healthcare, SWEDEN). The estimation of an apparent molecular weight for an unknown protein was performed by calibrating the size-exclusion column with standard proteins (thyroglobulin (MW: 670,000 Da), γ-globulin (MW: 160,000 Da), bovine serum albumin (MW: 69,000 Da), ovalbumin (MW: 45,000 Da), adenylate kinase (MW: 32,000 Da), myoglobin (MW: 17,000 Da), ribonuclease A (MW: 13,000 Da) and aprotinin (MW: 6,500 Da) with known molecular weight, size and compactness. A calibration plot was constructed for the respective column to determine the desired protein's apparent molecular weight. It was plotted with logarithms of molecular weights of the standard proteins versus their measured retention volumes (K_{av}). The K_{av} was calculated based on an equation, [(Elution volume, Ve) - (Void volume, Vo)]/[(Total volume, Vt) - (Void volume, Vo)]. The Rv1288 protein fractions were subjected to the 12% SDS-PAGE gel to check the purity of the protein.

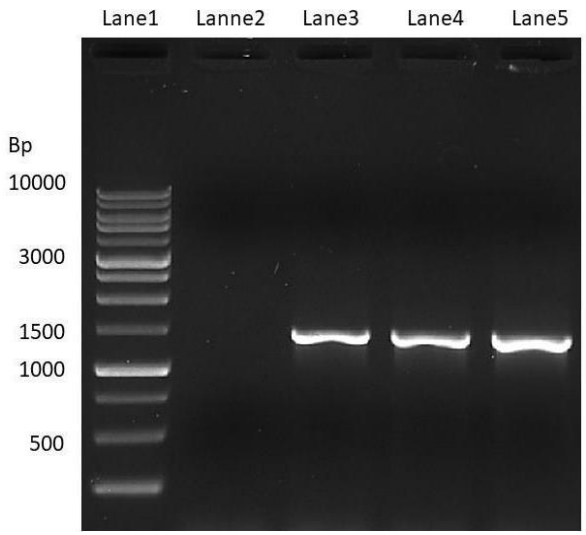

Figure 1: PCR products of the Rv1288 gene on 1% agarose gel. The gene was amplified by using 3 PCR steps, denaturation, annealing and extension, under an optimised condition. Lane 1: 1 kb ladder (GeneDirex, TAIWAN), Lane 2: Negative control, Lane 3-5: Amplified Rv1288 (~1400 bp).

RESULTS AND DISCUSSION

The *Rv1288* gene was successfully amplified at the optimised PCR condition with a final size product of ~1400 bp (Figure 1) and cloned into the *pET24d* vector to produce recombinant plasmids containing the gene of interest. Sequence analysis on the recombinant plasmid *Rv1288-pET24d* using T7 primers showed that the ligated *Rv1288* gene was at the correct orientation with its ends occupied by *Nco*I and *Xho*I sequences, respectively, and no mutation had been detected in the gene sequences. Sequence analysis using forward T7 primer is shown in Figure 2.

The full-length of the *Rv1288* gene sequence obtained from the sequencing analysis was translated into 456 amino acid residues which were identical to the residues obtained from the GenBank database. The size of the protein is 50.7 kDa, calculated by the ExPASy translation tool (Figure 3). The protein was successfully expressed in *E. coli* cells, induced with 1 mM IPTG at 20 °C with medium aggregation, 110 g for 48 h. The 12% SDS-PAGE analysis showed that the protein was expressed at the expected molecular weight (~51 kDa), the soluble and insoluble protein solution was estimated to be 40:60 and the amount of the protein was determined by using Bradford assay (Figure 4).

The Rv1288 protein was initially purified and fractionated by using the affinity column under a linear gradient of imidazole (0-70%) in Buffer B and the protein was fractionated and eluted from the column between a volume of ~45 mL to ~55 mL corresponding to

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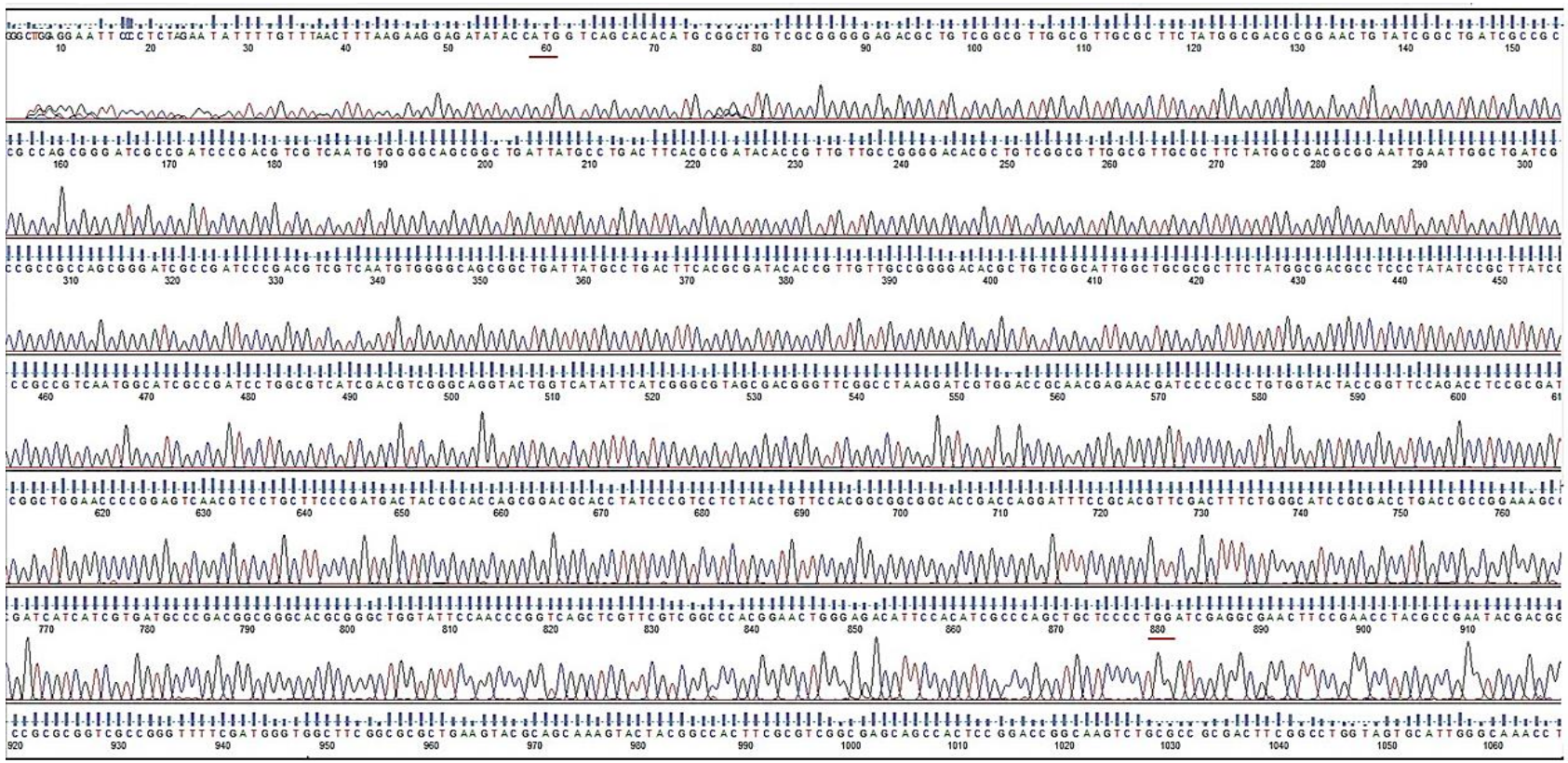

Figure 2: Sequencing analysis on the recombinant plasmid Rv1288-pET24d by using forward T7 primer. The analysis showed the presence of the Rv1288 gene in the plasmid at the correct orientation with the Start codon (ATG; underlined) from position (60-880) and no mutation was detected in the gene sequences.

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MVSTHAVVAG ETLSALALRF YGDAELYRLI AAASGIADPD VVNVGQRLIM
PDFTRYTVVA GDTLSALALR FYGDAELNWL IAAASGIADP DVVNVGQRLI
MPDFTRYTVV AGDTLSALAA RFYGDASLYP LIAAVNGIAD PGVIDVGQVL
VIFIGRSDGF GLRIVDRNEN DPRLWYYRFQ TSAIGWNPGV NVLLPDDYRT
SGRTYPVLYL FHGGGTDQDF RTFDFLGIRD LTAGKPIIIV MPDGGHAGWY
SNPVSSFVGP RNWETFHIAQ LLPWIEANFR TYAEYDGRAV AGFSMGGFGA
LKYAAKYYGH FASASSHSGP ASLRRDFGLV VHWANLSSAV LDLGGGTVYG
APLWDQARVS ADNPVERIDS YRNKRIFLVA GTSPDPANWF DSVNETQVLA
GQREFRERLS NAGIPHESHE VPGGHVFRPD MFRLDLDGIV ARLRPASIGA
AAERAD

Figure 3: Amino acid residues of *Mycobacterium Rv1288*. The total amino acid residues of the protein are 456 aa and the protein size was 50.7 kDa, calculated by the ExPasy translate tool.

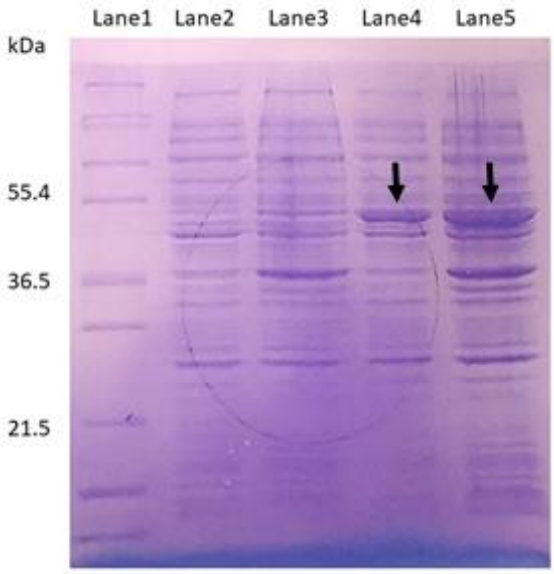

Figure 4: SDS-PAGE analysis of the protein expression of *Mycobacterium Rv1288*. The protein was expressed with the expected molecular weight of ~51 kDa (black arrows) at 20 °C for 48 h, 180 rpm. Lane 1: M12 protein marker, Lane 2: Pre-induced sample, Lane 3: Supernatant containing protein (CFE), Lane 4: Soluble *Rv1288* protein, Lane 5: Insoluble *Rv1288* protein. The SDS gel was soaked in Coomassie blue dye (BioRad, USA).

approximately of ~0.3 M imidazole (Figure 5A). In the second step of purification, 2 mg/mL Rv1288 protein was successfully fractionated and eluted in buffer A containing 0.5 M NaCl and Tris-HCl (1 M) (pH 8.0) by using AKTA pure chromatography aided with the size exclusion columns at an elution volume (Ve) of 50.9 mL (Figure 5B). Based on the SDS gel analysis, the purity of the protein was estimated at 90% (Figure 6). Given the calibrated void volume (Vo) and total volume (Vt) of the column were 41 mL and 75 mL, respectively, the measured retention volumes (K_{av}) value for the *Rv1288* protein

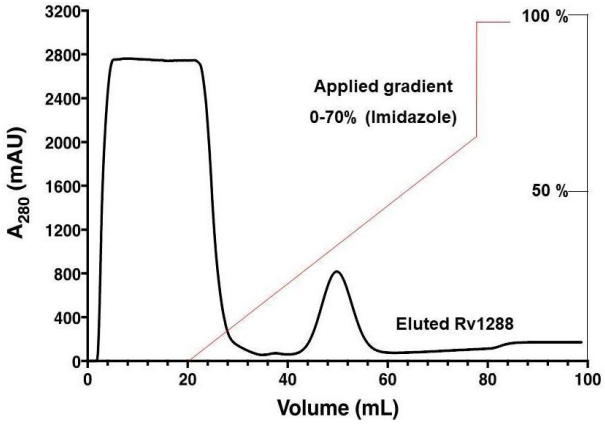

Figure 5A: The purification profile of the *Rv1288* protein by affinity chromatography utilising 5 mL HisTrap HP nickel affinity column. The protein was fractionated and eluted from the column between a volume of ~45 mL to ~55 mL, corresponding to approximately ~0.3 M imidazole.

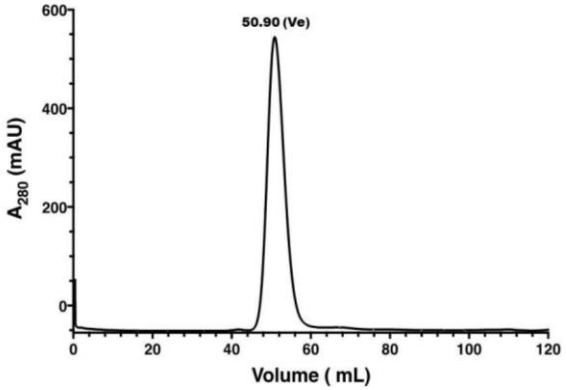

Figure 5B: The purification profile of the *Rv1288* protein by size exclusion chromatography (SEC) utilising a Superdex-200 pg column. The protein was fractionated and eluted in Buffer A at an elution column volume (Ve) of 50.9 mL.

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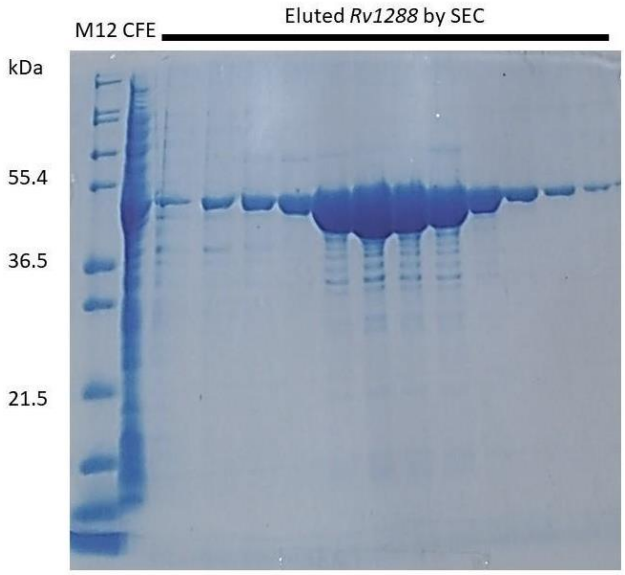

Figure 6: The SDS-PAGE gel of purified *Rv1288* (~51 kDa) by SEC. The purity of the final product was estimated to be ~90%. Lane 1: M12 protein marker, Lane 2: Cell-free extract (CFE), Lane 3-14: Fractionated and eluted soluble *Rv1288* from the Superdex-200 pg column obtained within elution volumes (Ve) 41 to 75 mL. The SDS gel was soaked in InstantBlue dye (Abcam, USA).

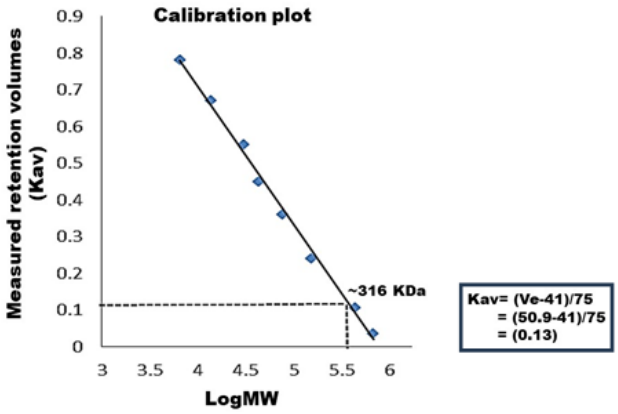

Figure 7: A calibration plot of the Superdex-200 pg column calibrated using eight standard proteins (Thyroglobulin, γ-Globulin, BSA, Ovalbumin, Adenylate kinase, Myoglobin, Ribonuclease A and Aprotinin). Based on the Ve value of 5.90 obtained from the purification profile, the calculated Kav of the Rv1288 protein was ~0.13, which gave the apparent molecular weight of the protein ~316 kDa. The equation for the Kav calculation is shown in the box.

~0.13 to give the final calculated apparent molecular weight of the Rv1288 protein ~316 kDa (LogMW of 5.5) (Figure 7). Given that the molecular weight of the protein was 50.7 kDa, it was suggested that Rv1288 was probably a hexamer in solution. To avoid protein aggregation, only a low concentration of protein is needed to ensure the association and dissociation of the protein happen at a high association constant (Tivol *et al.*, 1975; Engelborghs, 2012). The protein oligomerizes when it reaches stability at its equilibrium environments, and the state could also suggest how it interacts with other molecules intracellularly. The size-exclusion chromatography equipped with a gel filtration column with a specific size of pores is a conventional method to analyse size, compactness, and protein molecules interactions either between its species or other ligands (Wang *et al.*, 2010, Skaug *et al.*, 2015). Diffusion and aggregation of the proteins can be detected based on the asymmetrical flow of the latter by using a gel filtration column analysis under optimised conditions (Gu and Janson, 2001; Gabrielson *et al.*, 2007). However, other methods to further confirm the protein oligomerization in solution include ultracentrifugation (Heuberger *et al.*, 2002), NMR-microscopic and cryo-electron microscopy (Sborgi *et al.*, 2015). In this study, we report that the cloning, expression, purification and oligomerization analysis of *M. tuberculosis* Rv1288 was successfully performed to serve a further study on the investigation of its three-dimensional structure and functional studies (Brent *et al.*, 2017).

CONCLUSION

All the methods to produce and express the recombinant *Rv1288-pET24d* and its soluble protein in *E. coli* cells, respectively, have been successfully optimised and described here. Hence, it can be implemented for future studies to further investigate the biological properties of the protein at a molecular level as the information will shed light on the mechanism of the novel *M. tuberculosis* inhibitors and drug therapeutics design and development.

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CONFLICTS OF INTEREST

We would like to declare that there is no conflict of interest among the authors on the subject matter.

REFERENCES

Babaki, M. K. Z., Soleimanpour, S. and Razaee, S. A. (2017). Antigen 85 complex as a powerful *Mycobacterium tuberculosis* immunogene: Biology, immune-pathogenecity, applications in diagnosis and vaccine design. *Microbial Pathogenesis* **112, 20-29.**

Bateman, A. and Bycroft, M. (2000). The structure of a LysM domain from *E. coli* membrane-bound lytic

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

murein transglycosylase D (MltD)1. *Journal of Molecular Biology* **299(4), 1113-1119.**

Béliveau, C., Potvin, C., Trudel, J., Asselin, A. and Bellemare, G. (1991). Cloning, sequencing, and expression in *Escherichia coli* of a *Streptococcus faecalis* autolysin. *Journal of Bacteriology* **173(18), 5619-5623.**

Bolton, M. D., van Esse, H. P., Vossen, J. H., de Jonge, R., Stergiopoulos, I., Stulemeijer, I. J. E. *et al.* (2008). The novel *Cladosporium fulvum* lysin motif effector Ecp6 is a virulence factor with orthologues in other fungal species. *Molecular Microbiology* **69(1), 119-136.**

Brent, A. J., Mugo, D., Musyimi, R., Mutiso, A., Morpeth, S., Levin, M. *et al.* (2017). Bacteriological diagnosis of childhood TB: A prospective observational study. *Scientific Reports* **7, 11808.**

Desvaux, M., Dumas, E., Chafsey, I. and Hébraud, M. (2006). Protein cell surface display in Gram-positive bacteria: From single protein to macromolecular protein structure. *FEMS Microbiology Letters* **256(1), 1-15.**

Dubey, M., Vélëz, H., Broberg, M., Jensen, D. F. and Karlsson, M. (2020). LysM proteins regulate fungal development and contribute to hyphal protection and biocontrol traits in *Clonostachys rosea*. *Frontiers in Microbiology* **11, 679.**

Engelborghs, Y. (2012). An elegant way to quantitatively analyze oligomer formation in solution. *Biophysical Journal* **103(9), 1811-1812.**

Gabrielson, J. P., Brader, M. L., Pekar, A. H., Mathis, K. B., Winter, G., Carpenter, J. F. *et al.* (2007). Quantitation of aggregate levels in a recombinant humanized monoclonal antibody formulation by size-exclusion chromatography, asymmetrical flow field flow fractionation, and sedimentation velocity. *Journal of Pharmaceutical Sciences* **96(2), 268-279.**

Garvey, K. J., Saedi, M. S. and Ito, J. (1986). Nucleotide sequence of *Bacillus* phage Ø29 genes 14 and 15: Homology of gene 15 with other phage lysozymes. *Nucleic Acids Research* **14(24), 10001-10008.**

Gu, Z., Su, Z. and Janson, J. (2001). Urea gradient size-exclusion chromatography enhanced the yield of lysozyme refolding. *Journal of Chromatography A* **918(2), 311-318.**

Heuberger, E. H. M. L., Veenhoff, L. M., Duurkens, R. H., Friesen, R. H. E. and Poolman, B. (2002). Oligomeric state of membrane transport proteins analyzed with blue native electrophoresis and analytical ultracentrifugation. *Journal of Molecular Biology* **317(4), 591-600.**

Hu, S. P., Li, J. J., Dhar, N., Li, J. P., Chen, J. Y., Jian, W. *et al.* (2021). Lysin motif (Lysm) proteins: Interlinking manipulation of plant immunity and fungi. *International Journal of Molecular Sciences* **22(6), 3114.**

Kitaoku, Y., Fukamizo, T., Numata, T. and Ohnuma, T. (2017). Chitin oligosaccharide binding to the lysin motif of a novel type of chitinase from the multicellular green alga, *Volvox c*arteri. *Plant Molecular Biology* **93(1-2), 97-108.**

Liu, T., Liu, Z., Song, C., Hu, Y., Han, Z., She, J. *et al.* (2012). Chitin-induced dimerization activates a plant immune receptor. *Science* **336(6085), 1160-1164.**

Maan, P., Kumar, A., Kaur, J. and Kaur, J. (2018). Rv1288, a two domain, cell wall anchored, nutrient stress inducible carboxyl-esterase of *Mycobacterium tuberculosis*, modulates cell wall lipid. *Frontiers in Cellular and Infection Microbiology* **8, 421.**

Oguiza, J. A. (2022). LysM proteins in mammalian fungal pathogens. *Fungal Biology Reviews* **40, 114-122.**

Quinonez, C. G., Lee, J. J., Lim, J., Odell, M., Lawson, C. P., Anyogu, A. *et al.* (2022). The role of fatty acid metabolism in drug tolerance of *Mycobacterium tuberculosis*. *mBio* **13(1), e0355921.**

Radutoiu, S., Madsen, L. H., Madsen, E. B., Felle, H. H., Umehara, Y., Grønlund, M. *et al.* (2003). Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. *Nature* **425(6958), 585-592.**

Ruhland, G. J., Hellwig, M., Wanner, G. and Fiedler, F. (1993). Cell-surface location of *Listeria*-specific protein p60--detection of *Listeria* cells by indirect immunofluorescence. *Journal of General Microbiology* **139(3), 609-616.**

Sánchez-Vallet, A., Tian, H., Rodriguez-Moreno, L., Valkenburg, D. J., Saleem-Batcha, R., Wawra, S. *et al.* (2020). A secreted LysM effector protects fungal hyphae through chitin-dependent homodimer polymerization. *PLoS Pathogens* **16(6), e1008652.**

Sborgi, L., Ravotti, F., Dandey, V. P., Dick, M. S., Mazur, A., Reckel, S. *et al.* (2015). Structure and assembly of the mouse ASC inflammasome by combined NMR spectroscopy and cryo-electron microscopy. *Proceedings of the National Academy of Sciences of the United States of America* **112(43), 13237-13242.**

Shumayla, Madhu, Singh, K. and Upadhyay, S. K. (2022). LysM domain-containing proteins modulate stress response and signalling in *Triticum aestivum* L. *Environmental and Experimental Botany* **189, 104558.**

Skaug, M. J., Wang, L., Ding, Y. and Schwartz, D. K. (2015). Hindered nanoparticle diffusion and void accessibility in a three-dimensional porous medium. *ACS Nano* **9(2), 2148-2156.**

Steen, A., Buist, G., Kramer, N. E., Jalving, R., Benus, G. F. J. D., Venema, G. *et al.* (2008). Reduced lysis upon growth of *Lactococcus lactis* on galactose is a consequence of decreased binding of the autolysin AcmA. *Applied and Environmental Microbiology* **74(15), 4671-4679.**

Steen, A., Buist, G., Leenhouts, K. J., El Khattabi, M., Grijpstra, F., Zomer, A. L. *et al.* (2003). Cell wall attachment of a widely distributed peptidoglycan binding domain is hindered by cell wall constituents. *Journal of Biological Chemistry* **278(26), 23874-23881.**

Takayama, K., Wang, C. and Besra, G. S. (2005). Pathway to synthesis and processing of mycolic acids

in *Mycobacterium tuberculosis*. *Clinical Microbiology Reviews* **18(1), 81-101.**

Tivol, W. F., Beckman, E. D. and Benisek, W. F. (1975). Effect of protein concentration on the molecular weight of a5-3xetosteroid isomerase. *Journal of Biological Chemistry* **250(1), 271-275.**

Uhlén, M., Guss, B., Nilsson, B., Gatenbeck, S., Philipson, L. and Lindberg, M. (1984). Complete sequence of the staphylococcal gene encoding protein A. A gene evolved through multiple duplications. *Journal of Biological Chemistry* **259(3), 1695-1702.**

Visweswaran, G. R. R., Leenhouts, K., van Roosmalen, M., Kok, J. and Buist, G. (2014). Exploiting the peptidoglycan-binding motif, LysM, for medical and industrial applications. *Applied Microbiology and Biotechnology* **98(10), 4331-4345.**

Vollmer, W., Blanot, D. and de Pedro, M. A. (2008). Peptidoglycan structure and architecture. *FEMS Microbiology Reviews* **32(2), 149-167.**

Wang, Y., Teraoka, I., Hansen, F. Y., Peters, G. H. and Hassager, O. (2010). A theoretical study of the separation principle in size exclusion chromatography. *Macromolecules* **43(3), 1651-1659.**

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