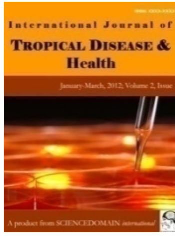

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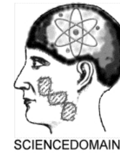

Rv2485c, a Putative Lipase of *M. tuberculosis*: Expression, Purification and Biochemical Characterization

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Authors' contributions

This work was carried out in collaboration between all authors. Author JK designed the study and the work was carried out under her supervision. Author GS managed the literature searches, performed the cloning, expression, purification and biochemical characterization of the gene, and wrote the first draft of the manuscript. Author SA designed and performed the gene expression in stress conditions and author M carried out the mutagenesis of the gene for active site residues and proof readed the manuscript. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Lipases have been demonstrated to have a role in virulence in several pathogens. Rv2485c gene product of *Mycobacterium tuberculosis* has been annotated as putative carboxyl esterase (LipQ) involved in cellular metabolism and respiration. The gene was expressed only in oxidative stress condition in *in vitro* culture of *M. tuberculosis* H37Ra as shown by Real Time PCR which suggests its role during dormant stage. Thereby, Rv2485c gene was cloned and expressed in *E. coli*. The LipQ enzyme was purified as a His-tagged protein from inclusion bodies and refolded with 37% protein yield. The specific activity of purified enzyme was calculated to be 93 U/mg with pNP-palmitate as a preferred substrate. It showed optimum enzyme activity in the range of 40-50^{0}C and pH 8.0. The Ser-249, Asp-344 and His-377, predicted as the member of the catalytic triad, were confirmed by site directed mutagenesis. The enzyme was inhibited in the presence of PMSF and DEPC suggesting the presence of Ser and His residues in catalytic site. The apparent K_m and V_{max} were calculated to be 1.45 mM & 196.08 U/ml respectively.

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The turnover number (k_{cat}) of the enzyme was calculated to be 6.597 min^{-1}. Based on the results it might be suggested that the LipQ is a lipase, hydrolyzing long chain esters, while the expression of gene only in oxidative stress condition suggested that the enzyme might be playing a role in intracellular survival of microorganism in the human macrophages. The manuscript deals with the detail characterization of oxidative stress inducible lipase and represents a step towards the elucidation of its biological function *in vivo.*

Keywords: Mycobacterium tuberculosis; lipase; pNP-palmitate; real time PCR; oxidative stress.

1. INTRODUCTION

Mycobacterium tuberculosis is an intracellular pathogen infecting primarily mononuclear phagocytes. The bacterium has developed intricate strategies to evade killing mechanisms of phagocytes [1]. A key to fight this disease is the identification and characterization of all these different strategies [2]. Lipolytic enzymes demonstrated several effects on macrophages i.e. inhibition of phagocytic functions as well as modulation of the release of inflammatory mediators [3].

Certain virulence factors are necessary for full pathogenicity of microorganism regardless of the host. The lipolytic enzymes were demonstrated as one of the known virulence factor in many bacteria such as *Pseudomonas cepacia*, *Staphylococcus aureus* [4,5] and in fungal species like *Alternaria brassicicola*, *Candida albicans*, and *Fusarium graminearum* [6-8]. Many different bacterial species produced lipases, which hydrolyzed esters of glycerol, preferably a long-chain fatty acids. They acted at the interface generated by a hydrophobic lipid substrate in a hydrophilic aqueous medium [9]. Despite lipolytic activities being described in mycobacteria many years ago, the associated enzymes, lipases/esterases and phospholipases, had only been the focus of intense research for the last few years. Because these enzymes were shared by several mycobacterial species, they represented a huge field of exploration not only for tuberculosis but also for other mycobacterial diseases [10]. Recently reported immunogenicity of mycobacterial lipases prompted its use as biomarkers for active tuberculosis [11]. On the other hand these enzymes were reported to be involved in the degradation of host lipids to provide fatty acids to pathogens [12].

Hence lipolytic enzymes might be considered as potent therapeutic targets for developing future anti-tuberculosis drugs. *M. tuberculosis* expresses a plethora of lipolytic enzymes, of which only a limited number had been studied so far and much remained to be explored for the diversity of their biological functions. It had been shown that during infection, *M. tuberculosis* accumulates intracellular lipid inclusions (ILI) that were probably derived from lipids of the host cell membrane. LipY has been found to be responsible for the utilization of accumulated TG (triglycerides) during dormancy and reactivation of the pathogen [13]. Deletion mutant of LipY, with compromised ability to mobilize the stored TG, but not the complemented mutant, was unable to come out of dormancy upon treatment with anti-TNFα mAbs [14]. The overproduction of LipY in mycobacteria resulted in a significant reduction in the pool of TAGs (triacylglycerols), consistent with the lipase activity of this enzyme [15]. The gene product of Rv3487c (LipF) has been cloned and characterized, but no long-chain TG hydrolysis was detected [16]. On the basis of structural genomic approach, cloning and biochemical characterization of Rv1399c gene product, LipH was carried out [17]. Its biochemical behavior univocally classified Rv1399c as a non-lipolytic rather than a lipolytic hydrolase. However, at present the physiological roles for many of these lipases had not

been completely deciphered. As it was already described that in latent state, the lipids are the main source of energy to the starving pathogen, therefore, all the lipid hydrolyzing enzymes of *M. tuberculosis* should be characterized to ascertain their role in survival/patogenicity/virulence.

LipQ enzyme was predicted to be involved in intermediary metabolism and demonstrated 66% similarity with its counterpart in non-pathogenic strain *M. smegmatis,* while no change in gene sequence was noticed in *M. tuberculosis* H37Ra strain. The effect of Rv2485c gene product on activation of murine macrophages was investigated in lab (unpublished results). No cytotoxicity was observed after treatment of cells with LipQ protein (unpublished results). The LipQ protein inhibited LPS induced nitric oxide (NO) production by macrophages. Decreased NO production could be directly correlated with inhibition of iNOS mRNA expression in murine macrophage (unpublished results). These observations suggested the probable role of this enzyme in survival of mycobacteria in the mature granulomas during the dormant phase. Therefore in the present investigation Rv2485c gene, GenBank number: 887876 from *M. tuberculosis* H37Rv was cloned and expressed in *E. coli*. The detail biochemical characterization of LipQ protein was carried out. The expression of Rv2485c gene was checked in normal *in vitro* culture of *M. tuberculosis* H37Ra in different phases of growth and under various stress conditions.

2. MATERIALS AND METHODS

2.1 Databases and Software

The DNA sequences of the Rv2485c gene was retrieved from the NCBI GenBank. Online BLAST (http://blast.ncbi.nlm.nih.gov) was employed for analyzing the homologous identity of the Rv2485c of *M. tuberculosis* with other mycobacterial species [18]. The identical protein sequences of the Rv2485c in different species were aligned with Clustal X1.83 (http://www.clustal.org/) and all the positions containing gaps were eliminated [19]. By using software Phyre [20] (Imperial college, London), secondary structure was predicted. The *M. tuberculosis* H37Rv chromosomal DNA was a kind gift from Dr V. M. Katoch, JALMA, Agra, UP, India.

2.2 Cloning of Rv2485c Gene in *Escherichia coli*

The Rv2485c coding region from *M. tuberculosis* H37Rv chromosomal DNA was amplified using the primers GSF2 and GSR2 (Table 1).

Table 1. Primer sequence used to clone the Rv2485c gene

Primer name	Sequence
GSF2	5'-tgcacatcgccagcgtgactt-3’
GSR2	5’-gaacggccaagctcagct -3’

The thermocycler (Perkin Elmer Cetus, Foster City, CA, U.S.A.) was programmed for a hot start of 94ºC for 4 min followed by 30 cycles at 94ºC for 30 sec, 60ºC for 40 sec and 72ºC for 2 min and a final cycle of 7 min at 72ºC. The amplified and purified DNA was ligated to the pQE30-UA vector (Qiagen, MD, USA) to generate construct pQE-LipQ. *E. coli* M15 (PREP4) was used for the transformation and expression of Rv2485c. The transferred *E. coli* strain was grown in Luria-Bertani (LB) medium with kanamycin (30 µg/ml) and ampicillin (100

µg/ml). The presence of Rv2485c gene was confirmed by plasmid isolation [21], colony PCR and sequencing.

2.3 Expression and Purification of LipQ

For shake flask growth, 1 liter of Luria broth medium supplemented with kanamycin (30 µg/ml) and ampicillin (100 µg/ml) was inoculated with 10 ml of an overnight culture and incubated at 37^0C. The culture was grown upto midlog phase (A_{600}~0.6), induced with 0.5 mm isopropyl thio-β-d- galactosidase (IPTG), and further incubated for 3 h at 37ºC.

Cells were harvested at 4ºC by centrifugation at 9,000 g for 15 min and resuspended in cold lysis buffer (50 mM TrisCl, pH 8.0, 0.1% (v/v) triton X-100, 150 mM NaCl and 1 mM EDTA) at 5 ml/g wet weight and stored overnight at -80^0C. The cell suspension was thawed on ice for 1 h and 20 mM $MgSO_4$ (final concentration) was added. Cells were disrupted by ultrasonication (10× with a 15 s cycle) using a Pharmacia Biotech Sonifier. Cell debris were separated from the cell extract by centrifugation at 14, 000 g for 20 min. The pellet was solubilized by stirring at 4ºC overnight in a 40 ml solution containing 10 mM TrisCl, pH 8.0, 100 mM sodium dihydrogen phosphate and 8 M urea. The inclusion bodies were separated from solubilized protein by centrifugation at 14, 000 g for 20 min at 4ºC. Recombinant LipQ was purified from solubilized inclusion bodies by metal affinity chromatography under denaturing conditions using a nickel nitrilotriacetic acid (NiNTA) column as described by the manufacturer (Qiagen). Solubilized inclusion bodies were loaded on a nickel nitrilotriacetic acid column previously equilibrated with equilibration buffer (10 mM TrisCl, pH 8.0, 100 mM sodium dihydrogen phosphate and 8 M urea). After that protein loading column was washed with equilibration buffer at pH 6.3, and bound protein was eluted using a pH gradient (pH 6.3 - 4.3) of equilibration buffer. The fractions of eluted peak containing purified LipQ were checked for presence of protein after dilutions by measuring absorption at 280 nm with prepared blank. The protein was also analyzed by SDS-PAGE [21] on a 12.5% gel that was subsequently stained with Coomassie brilliant blue.

2.4 Refolding LipQ by Dialysis

Refolding was carried out with chaotropic agent for concentration gradient dialysis. The solution of denatured protein (1.5 mg/ml) was dialyzed against 2 L of freshly made 6, 4, 2, 1, 0.5, and 0 M urea, respectively, in 10 mM sodium phosphate buffer (pH 8.0). With each concentration, the protein was dialyzed 6 h at 4^0C. Protein concentration was determined by Protein estimation kit (BCA method) procured from Bangalore GeNei.

2.5 Lipase Assay

The enzyme activity was determined according to the modified method of Sigurgisladottirr et al. [22]. Hundred microliter enzyme (100 mg/ml) and 0.1 ml of 0.002 M pNP-palmitate was added in 0.8 ml of 0.05 M phosphate buffer (pH 8.0). The reaction was carried out at 37^0C for 15 min, after which 0.25 ml 0.1 M Na_2CO_3 was added. The mixture was centrifuged and the activity was determined by measuring absorbance at 420 nm. One unit of enzyme activity is defined as the amount of enzyme, which liberates 1 µmole of p-nitrophenol from pNP-ester as substrate per min under standard assay conditions.

2.6 Biochemical Properties of Rv2485c

2.6.1 Effect of pH and temperature on enzyme activity/stability

Lipase activity was assayed in the buffers of different pH (2.0-10.5) at 37ºC. The following buffers (50 mM) were used: Glycine/Hydrochloric acid, pH 2.0-3.0; acetate buffer, pH 4.0–5.5; phosphate buffer, pH 6.0–8.0; Tris/Cl buffer, pH 8.0–9.0 and carbonate bicarbonate buffer, pH 10.0–10.5. The pH stability of the lipase was determined by incubating the enzyme at different pH for 1 h at room temperature. The residual lipase activity was determined by standard assay method at pH 8.0.

The effect of temperature on enzyme activity was determined by carrying out the enzyme assay at different temperatures (20-90ºC). Thermal stability of LipQ was determined by incubating enzyme at different temperatures (20-90ºC) for 30 min and pH 8.0 followed by cooling of enzyme on ice for 15 min. Enzyme activity was measured as per standard assay conditions.

2.7 Substrate Specificity

The substrate specificity of the purified recombinant protein was measured using pNP esters (Sigma, USA) with carbon chain length ranging from C_4 to C_{18}. The released pNP from the substrates *p*-nitrophenyl-butyrate (pNPB; C_4), *p*-nitrophenyl-decanoate (pNPD; C_{10}), *p*-nitrophenyl-laurate (pNPL; C_{12}), *p*-nitrophenyl-myristate (pNPM; C_{14}), *p*-nitrophenyl-palmitate (pNPP; C_{16}) and *p*-nitrophenyl-stearate (pNPS; C_{18}) were monitored using spectrophotometric assay.

2.8 Enzyme Kinetics

The effect of pNP-palmitate concentration (0.1- 5 mM) on the reaction rate of the enzyme was measured by using standard enzyme assay. The Michaelis–Menten constant (K_m) and maximum velocity for the reaction (V_{max}) were determined by Lineweaver–Burk plot and k_{cat} (turnover number) & k_{cat}/K_m (specific constant) were calculated.

2.9 Site-Directed Mutagenesis

To confirm the predicted active site residues of this enzyme, site-directed mutagenesis of Ser-249, Asp-289, Asp-344 and His-377 of LipQ was carried out using a pair of complementary primers (Table 2) containing the desired mutations by a non-strand-displacing, high-fidelity polymerase (New England Biolabs) in a linear amplification. (The amino acids were changed as followed: Ser249 - Ala249, Asp289 - Ala289, Asp344 - Ala344, His377 - Pro377).

Table 2. Primer sequence used to clone site directed mutants of the Rv2485c gene

Primer name	Sequence
t745g	5'-caccggcggggccgccggtgc-3'
t745g_antisense	5'-gcaccggcggccccgccggtg-3'
a866c	5'-tacggcgtctacgccctcaccaacgcc-3'
a866c_antisense	5'-ggcgttggtgagggcgtagacgccgta-3'
a1031c	5'-tgttgcacggtgagaaggctccgatggtgc-3'
a1031c_antisense	5'-gcaccatcggagccttctcaccgtgcaaca-3'
a1130c	5'-ccaacgcccaccccgcgttcgacct-3'
a1130c_antisense	5'-aggtcgaacgcggggtgggcgttgg-3'

The clones were sequenced to confirm the mutation. The mutant enzymes were expressed, purified and refolded along with the native protein. The activity of enzyme was determined as per standard assay method.

2.10 Effect of Various Additives on Enzyme Activity

The effect of various lipase /esterase inhibitors phenylmethanesulfonylflouride (PMSF), chemical modifier diethylpyrocarbonate (DEPC) and beta mercaptoethanol (β-ME) (0.1 and 1.0 mM) were studied with reference to enzyme activity of LipQ. The enzyme and additives were incubated at room temperature for 5 min prior to assay. The enzyme without any additive served as 100%. Additionally in the case of PMSF and DEPC (1 mM concentration), the enzyme and PMSF or DEPC was incubated at 50ºC for 5 min prior to assay. Then enzyme assay was performed according to standard assay method.

2.11 Growth of *M. tuberculosis* H37Ra

M. tuberculosis H37Ra culture was grown in middlebrook 7H9 broth base (Himedia) supplemented with 1 % glycerol and 0.05 % Tween-20. An additional 1 % (v/v) growth supplement OADC (BBL) was added. The cells were grown for 7 days upto mid log phase (A_{600} 0.7-0.8) and harvested by centrifugation. The bacterial pellet was resuspended in different media for exposure to stress conditions such as oxidative stress (7H9 growth media with 5 mM H_2O_2) [23] acidic stress (7H9 growth media at pH 4.5) [24] and nutrient stress (1X phosphate buffer saline) [25]. These three stressed cultures were grown for 6 h, along with control culture in middlebrook 7H9 broth base (normal pH, without any additive). Samples were also taken out at mid log phase and stationary phase (A_{600} 1.8-2). RNA was isolated from all the samples using TRI reagent from Sigma as per company's manual instructions.

2.12 Rv2485c Expression Analysis

The RNA samples were converted into complementary DNA (cDNA) using RevertAid™ cDNA kit (Fermentas). This cDNA was used as a template to check the relative expression of the Rv2485c gene. 16S RNA was used as an internal control (Table 3). Expression pattern of Rv2485c gene was analyzed in acidic, nutritional, and oxidative stress and mid log and stationary phase in *in vitro* culture of *M. tuberculosis* H37Ra using short primers, sGSF2 as forward and sGSR2 as reverse primer (Table 3). The specificity of the reactions was verified by melting curve analysis. Determination of expression level of the gene was carried out by using Real-Time Quantitative PCR and the $2^{-\Delta\Delta Ct}$ Method [26]. Ct values for each run were used to calculate mean Ct with SD (Standard Deviation). For each sample: ΔCt test=

Ct test- Ct 16S rRNA, ΔCt control= Ct control- Ct 16S rRNA. ΔΔCt values relative to control were calculated by following formula: ΔΔCt = ΔCt test – ΔCt control. The fold change in test gene expression was determined by taking 2 to the power of ΔΔCt values i.e $2^{-\Delta \Delta Ct}$.

Table 3. Short Rv2485c gene primer sequence and 16s RNA primer sequence used in the expression analysis

Primer name	Sequence
sGSF2	5'- acctccgacatcccgtac-3'
sGSR2	5'- ttgtaccgccgtgtcggc- 3'
16sRNA fwd	5'-gaggaaggtggggatgacgt-3'
16sRNA rev	5'-aggcccgggaacgtattcac-3'

3. RESULTS

3.1 Rv2485c Expression Analysis

The expression of Rv2485c gene of *M. tuberculosis* H37Ra was analyzed in mid log and stationary phase samples and also in acidic stress, oxidative stress and nutrient depletion by real-time PCR. No expression of Rv2485c was observed in log phase, stationary phase or stress conditions of bacterial growth by semi quantitative RT-PCR (data not shown). Therefore the expression was further checked by real time PCR. RNA of the control culture was used as a positive calibrator for real-time quantitative PCR. The specificity of the reactions was verified by melting curve analysis. Out of all the three stress condition, there was upregulation of expression of Rv2485c in oxidative stress only. The expression of Rv2485c was increased about 3.14 fold during oxidative stress and it was slightly down regulated in acidic sample in comparison to control samples. In case of nutrient sample there was not much difference than the control samples (Table 4, Fig. 1).

Table 4. The expression analysis of Rv2485c under normal and stress conditions by real time- PCR

Type stress	Ct value (test)	Ct value (16S RNA)	Δ Ct (test – 16 S)	ΔΔ Ct (Δ Ct test – Δ Ct control)	Fold change ($2^{-\Delta\Delta Ct}$)
Control	14.00	11.34	2.66	0	1
Acidic	16.00	11.34	4.66	2.00	0.25
Oxidative	13.50	12.50	1.00	-1.66	3.14
Nutrient	12.50	10.30	2.19	-0.47	1.386

All the values represented are mean ± SD of three independent experiments carried out in triplicate.

The DNA sequences of the Rv2485c gene was compared with homologous sequences of other species of mycobacteria. It showed least identity with β-lactamase of non-virulent strain, *M. smegmatis* (Table 5).

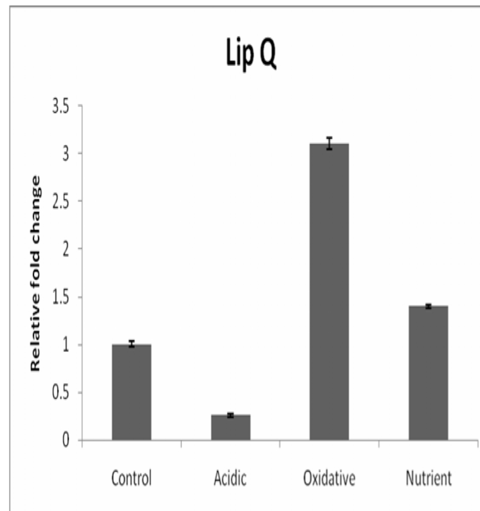

Fig. 1. Relative gene expression analysis for Rv 2485c. Values are the average results from 3 independent assays in triplicates, with error bars indicating standard deviations

Table 5. Percentage identity of Rv2485c nucleotide sequence with other species of *Mycobacterium* by using BLAST

Gene product	*M. smegmatis*	*M. marinum*	*M. bovis*
Rv2485c (LipQ)	66% with peptidase	76% with LipQ	100% with putative carboxylesterase

3.2 Cloning of Rv2485c

Rv2485c gene of *M. tuberculosis* H37Rv, with its original start codon, was amplified using chromosomal DNA as template. The amplified product corresponded with the expected size (1266 bp) of Rv2485c gene of *M. tuberculosis* H37Rv. The amplified fragments were purified and ligated into pQE30-UA vector and transformed into competent *E. coli* M15 cells carrying *lacI* plasmid pREP4. Transformants were confirmed for the presence of Rv2485c insert by colony PCR. The sequencing results showed 100% homology with Rv2485c nucleotide sequence present in the complete genome sequence of *M. tuberculosis* H37Rv [27].

3.3 Purification of LipQ

The enzyme could not be expressed as soluble protein by varying the induction conditions. Purification of the urea solubilized enzyme, using affinity chromatography on Ni-NTA column as major method, yielded purified enzyme with 37% protein yield. Approximately 6 mg of pure and active recombinant enzyme was recovered from 1 liter of culture. The protein migrated as a single band on SDS-PAGE with an approximate molecular mass of 47.35 kDa (Fig. 2). The molecular mass was little higher than the calculated mass from amino acid sequence which agreed with the calculated molecular mass obtained from the sequence data plus the mass of the amino terminus-appended $(His)_6$ tag (2.1 kDa).

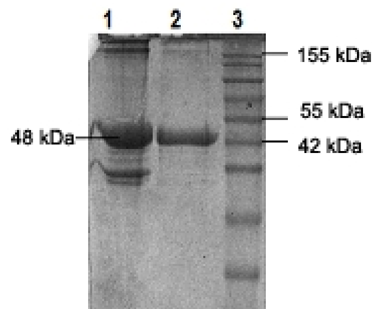

Fig. 2. SDS-PAGE analysis of purified LipQ. Protein samples were loaded on 12% SDS polyacrylamide gel under reducing conditions. Lane 1 - Lysate, Lane 2- purified LipQ and Lane 3- protein molecular weight marker

3.4 Bioinformatics Analysis

The theoretical *pI* of the LipQ protein was calculated to be 8.98 by using online tool ScanSite pI/Mw. The molecular weight of the protein was predicted to be 47.35 kDa. The protein was rich in hydrophobic amino acids (31%). It had 49.69% negatively charge amino acids and 31.15% positive charged amino acids and net charge at pH 7.0 was calculated to be 6.29. The secondary structure (SOPMA) predicted revealed that the protein consisted of 47.51% α-Helix, 10.45% extended strand, 4.51% β-turn and 37.53% random coils. Multiple sequence alignment of LipQ from *M. tuberculosis* and five other bacteria including *Bacillus* showed all these three active site amino acid residues are conserved (Supplementary Fig. 1).

3.5 Biochemical Characterization of Rv2485c

3.5.1 Substrate specificity

The enzyme was active on broad range of substrates (C_{10}-C_{18}). The maximum enzyme activity (100%) was observed with pNP-palmitate. The specific activity of enzyme was calculated to be 93 U/mg with pNP-palmitate as a preferred substrate. The result demonstrated the substrate choice for the recombinant lipase was in the order $C_{16}>C_{18}>C_{14}>C_{12}>C_{10}>C_4$ (Fig. 3).

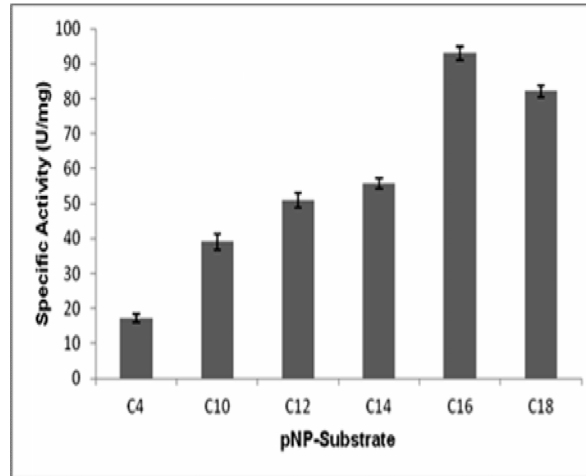

Fig 3. Substrate specificity of recombinant LipQ. The reaction mix without enzyme served as blank. Values are the average results from 3 independent assays in triplicates, with error bars indicating standard deviations

3.6 Effect of Temperature and pH on Enzyme Activity

The enzyme was active over wide range of temperature i.e. 30-50ºC having temperature optimum at 40ºC with pNP-palmitate as substrate. Nearly 45% activity was observed at 20ºC and 60ºC. The enzyme was stable from 20ºC to 30ºC for 30 min; however it could retain nearly 70 & 60% activity at 40 & 50ºC respectively (Fig. 4A). The enzyme was completely stable at 50ºC for 15 minutes; however the activity was reduced to 50% after 45 min incubation. The enzyme displayed maximum activity at pH 8.0 and was completely stable at pH 7.0 and nearly 90% stable at pH 6.0 & 8.0 at room temperature for 1h (Fig. 4B).

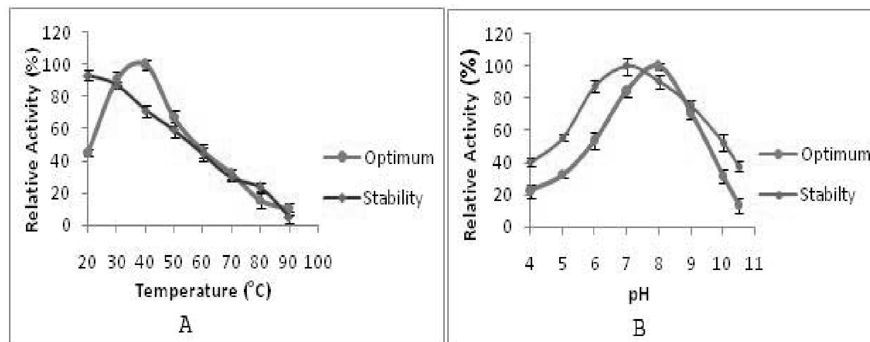

Fig. 4. Temperature (A) and pH (B) dependence activity and stability of LipQ. Values are the average results from 3 independent assays in triplicates, with error bars indicating standard deviations

3.7 Effect of Different Additives on Rv2485c Activity

PMSF and DEPC (1.0 mM v/v) inhibited the enzyme activity to nearly 60% and 80% loss in enzyme activity respectively. However the enzyme did not show any activity with 1.0 mM of

PMSF and DEPC after pre-incubation at 50^0C for 5 min (data not shown). Eserine did not affect the enzyme activity. The decline in enzyme activity was observed on treatment with β-ME (Fig. 5).

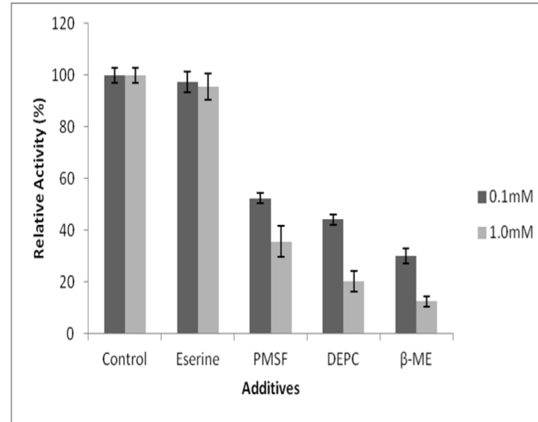

Fig. 5. Effect of various additives/chemical modifiers on Rv2485c activity. The experiment was repeated three times in triplicate with error bars indicating standard deviations

3.8 Kinetic Properties

The reaction catalyzed by LipQ (100 µl of 190 U/ml enzyme stock) was studied by monitoring the increase in released pNP absorbance at 420 nm. From the kinetic analysis, it was determined that the enzyme displayed maximum velocity of reaction at 1.0 mM concentration after which no further increase in velocity of the enzyme reaction was observed. The double-reciprocal plot (Lineweaver-Burk plot) of reaction rate was used to evaluate the Michaelis-Menten constant, K_m and maximum velocity, V_{max}. The apparent K_{m} and V_{max} values for substrate, pNP-palmitate was 1.45 mM & 196.08 U/ml. The turnover number (k_{cat}) of the enzyme was calculated to be 6.597 min^{-1}.

3.9 Structure Prediction and Identification of Active Site Residues

The Ser-249, Asp-344 and His-377 were predicted as the member of the catalytic triad. There were two Asp (one at 289 and another at 344 positions) residues found to be conserved in multiple sequence alignment (Supplementary Fig. 1). The protein structure of LipQ was predicted by homology modeling by using online software, Phyre with template, esterase EstE1 cloned by metagenomics [28] and image was produced and labeled by PyMol [29] (Supplementary Fig 2). Serine residue was found to be buried in the hydrophobic core. It suggested the chances of Asp 344 to be the active site residue. The active site residues of LipQ were confirmed by site-directed mutagenesis. For each of the altered proteins of Serine and Histidine residues, the enzyme activity was lost (~95%) with pNP-palmitate as substrate. Nearly 75% loss of enzyme activity was observed for Asp-344 mutant. More than 85% enzyme activity was retained with Asp-289 mutant.

4. DISCUSSION

Pathogenic bacteria utilize a number of mechanisms to cause disease in human hosts. Bacterial pathogens express a wide range of molecules that bind host cell targets to facilitate a variety of different host responses. The development of novel drugs against *M. tuberculosis* has numerous hurdles like identification of therapeutically relevant metabolic targets and introduction of favorable physiochemical characteristics.

The previous studies demonstrated that bacteria grown *in vitro* had a preference for carbohydrates, whereas bacteria grown *in vivo* preferred fatty acids as their carbon source giving due importance to lipolytic enzymes [30]. The bioinformatics study revealed the presence of lip family genes i.e. 24 putative lipases in *M. tuberculosis* H37Rv [31]. Present knowledge about the structure, biochemical properties, regulation and physiological role of all lipase enzymes of mycobacterium is still low. In some recent studies, few lipolytic enzymes of *M. tuberculosis* were characterized both biochemically and enzymatically [32] allowing us to propose that these enzymes, are very likely to participate in lipid metabolism during dormancy and/or during reactivation [33]. This view is supported by a recent study conducted by Low *et al.* in 2009 demonstrating extensive accumulation and degradation of TAGs in the bacilli during entry into and exit from hypoxia-induced dormancy, respectively [34]. A lip family gene, *LipF* has already been shown its role in pathogenesis [16]. Still lots of putative lipases/esterases remained to be studied in detail. Therefore, this study aims to characterize LipQ that was predicted to play some role in metabolism and respiration. Till date no reports were available on biochemical properties of LipQ as catalyst and their effects on host cells.

After purification and refolding only 37% of protein could be recovered as active protein due to aggregation problem. It was observed that LipQ exhibited more activity towards the long carbon chain substrates in comparison to short carbon chain substrates. It displayed maximum activity towards pNP-palmitate (C_{16}), at pH 8.0. It might be hypothesized that this enzyme might hydrolyze the palmitate present in mammalian cells to provide energy and might allow mycobacteria to survive for a long period of time. However in present investigation LipQ did not trigger membrane lysis when incubated with macrophage cells, thus displaying no cytotoxic effects on RAW 264.7 cells (data not shown). In contrast, Rv3452, a phospholipase A2, was reported to induce macrophage lysis [35].

The Lip Q was demonstrated to show very low activity with long chain TG hydrolase [13]. In similar studies with other putative lipases, no long-chain TG hydrolysis was detected with LipF [16]. On the other hand LipH, previously annotated as a putative lipase, efficiently hydrolyzed soluble triacylglycerols and vinyl esters but was inactive against emulsified substrate [17].

The possible catalytic triad of the lipase was predicted by multiple sequence alignment (Supplementary Fig. 1). LipQ showed presence of conserved pentapeptide, G-X-S-X-G motif (GGSAG) at positions 247-251, a conserved His at position 377 and two conserved aspartic acids at position 289 and 344.To further shed lights on the active site residues of this lipase, the enzyme was treated with PMSF and DEPC. The partial inhibition of enzyme with PMSF and DEPC at room temperature suggested that the active site residues appeared to be buried. Although the optimum temperature of the protein is 40ºC but it was preincubated at 50ºC with PMSF and DEPC to make the active site accessible to inhibitors because in many cases the proteins active site may be buried inside a hydrophobic core. The protein itself did

not loose much activity after incubation at 50ºC for 5 min (more than 95% activity retained, data not shown).

The inhibition of enzyme activity with PMSF and DEPC suggested the involvement of Ser and His residues in catalytic site. LipQ had five cysteine residues and predicted to have two disulphide bonds. Inhibition of LipQ enzyme activity with β-ME suggested the importance of S-S bonds in maintaining the proper conformation of enzyme.

It was reported earlier that the *M. tuberculosis* resides in macrophage compartment which has pH range from 6.2 to 4.5. This depends on the activation state of the macrophage [36]. *In vitro* assays carried out for the purified lipases showed significant activity in acidic pH range as well as enzyme could retain approximately 50% of enzyme activity at pH 5.0. However no noticeable up-regulation of Rv2485c transcription was observed during *in vitro* culture of *M. tuberculosis* H37Ra during acidic stress. It suggested that the promoter of this gene was not induced by acidic pH and therefore the protein was not specifically synthesized during acidic stress. On the other hand irrespective of the mode of synthesis, the enzyme might work during acidic stress condition as it was stable (retaining 90% enzyme activity) at pH 6.0. While the mechanisms by which *M. tuberculosis* responds to pH are poorly characterized, the global transcriptional response of *M. tuberculosis* to acid stress points to several regulators. In our study the enzyme was specifically induced during oxidative stress conditions only suggesting its role in dormant stage.

During initial infection the internal temperature of the host will be slightly higher than healthy patients. It is due to the defense mechanism of the host against the pathogen [37]. During this condition, the proteins, active at high temperature were predicted to play important role for the survival of pathogens [38]. During initial infection, temperature of the host is unlikely to soar beyond 45ºC, activity of LipQ at 50ºC might help not only to prevents its inactivation but ensures its functionality at this temperature. It was reported that the thermostability associated with Rv2416c protein play significant role in intracellular survival within human macrophages [39].

The idea that lipases and phospholipases could be involved in pathogenesis has been suggested by the results of several studies [40-41]. The purified lipases/phospholipases from these organisms demonstrated various response in eukaryotic cells *in vitro*. They inhibited the phagocytic function of alveolar macrophages and modulate the release of inflammatory mediators by various kinds of cells in the immune system [41-42].

5. CONCLUSION

In the present study LipQ, a putative lipase, was demonstrated to be a lipase hydrolyzing long chain esters while eserine, an esterase inhibitor, did not show any effect on enzyme activity. Biochemical parameters provide a clue about the environment where it may work efficiently. Rv2485c gene was expressed only in oxidative stress conditions and might help in fighting the oxidative damage caused by reactive oxidative species (ROS) during the dormant stage and also during the reactivation of the disease. Although the nature of LipQ substrate in *in-vivo* conditions and the role of this enzyme in the life cycle of *M. tuberculosis* remain to be investigated, this study represents some important clues towards the elucidation of the biological function of LipQ.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Ting LM, Kim AC, Cattamanchi A, Ernst JD. *Mycobacterium tuberculosis* inhibits IFN-gamma transcriptional responses without inhibiting activation of STAT1. J Immunol 1999;163:3898-3906.
2. Wilson J, Schurr M, LeBlanc C, Ramamurthy R, Buchanan K, Nickerson C. Mechanisms of bacterial pathogenicity. Postgrad Med J 2002;78:216-224.
3. Flynn JL, Chan J. Immunology of tuberculosis. Annu Rev Immunol 2001;19:93-129.
4. Lonon MK, Woods DE, Straus DC. Production of lipase by clinical isolates of *Pseudomonas cepacia*. J Clin Microbiol 1988;26:979-984.
5. Rollof J, Braconier JH, Soderstrom C, Ehle PN. Interference of *Staphylococcus aureus* lipase with human granulocyte function. Euro J Clin Microbiol Infect Dis. 1988;7:505-510.
6. Berto P, Commenil P, Belingheri L, Dehorter B. Occurrence of a lipase in spores of *Alternaria brassicicola* with a crucial role in the infection of cauliflower leaves. FEMS Microbiol Lett. 1999;180:183-189.
7. Tsuboi R, Komatsuzaki H, Ogawa H. Induction of an extracellular esterase from *Candida albicans* and some of its properties. Infect Immun 1996;64:2936-2940.
8. Voigt CA, Schafer W, Salomon S. A secreted lipase of *Fusarium graminearum* is a virulence factor required for infection of cereals. Plant J. 2005;42:364-375.
9. Andrejew A, Desbordes J. Hydrolysis of Fatty Acids Esters by *Mycobacterium phlei*. Ann Inst Pasteur. 1969;117:486–500.
10. Dedieu L, Serveau-Avesque C, Kremer L, Canaan S. Mycobacterial Lipolytic Enzymes: a Gold Mine for Tuberculosis Research. Biochimie in press. 2012 doi: 10.1016/j.biochi.2012.07.008.
11. Brust B, Lecoufle M, Tuaillon E, Dedieu L, Canaan S, Valverde V, Kremer L. *Mycobacterium tuberculosis* lipolytic enzymes as potential biomarkers for the diagnosis of active tuberculosis. PLoS ONE. 2011;6:p.e25078.

12. Daniel J, Maamar H, Deb C, Sirakova TD, Kolattukudy PE. *Mycobacterium tuberculosis* Uses Host Triacylglycerol to Accumulate Lipid Droplets and Acquires a Dormancy-Like Phenotype in Lipid-Loaded Macrophages. PLoS Pathog. 2011;6:7.
13. Deb C, Daniel J, Sirakova TD, Abomoelak B, Dubey VS, Kolattukudy PE. A novel lipase belonging to the hormone-sensitive lipase family induced under starvation to utilize stored triacylglycerol in *Mycobacterium tuberculosis*. J Biol Chem. 2006;281:3866-3875.
14. Kapoor N, Pawar S, Sirakova TD, Deb C, Warren WL, Kolattukudy PE. Human Granuloma *In Vitro* Model, for TB Dormancy and Resuscitation PLoS One. 2013;8(1):e53657.
15. Mishra KC, Chastellier CD, Narayana Y, Bifani P, Brown AK, Besra GS, Katoch VM, Joshi B, Balaji KN, Kremer L. Functional Role of the PE Domain and Immunogenicity of the *Mycobacterium tuberculosis* Triacylglycerol Hydrolase LipY. Infect Immun 2008;76(1):127-140.
16. Zhang M, Wang JD, Li ZF, Xie J, Yang YP, Zhong Y, Wang HH. Expression and characterization of the carboxyl esterase Rv3487c from *Mycobacterium tuberculosis*. Protein Expr Purif 2005;42:59-66.
17. Canaan S, Maurin D, Chahinian H, Pouilly B, Durousseau C, Frassinetti F, Calvo LS, Cambillau C, Bourne Y. Expression and characterization of the protein Rv1399c from *Mycobacterium tuberculosis*. A novel carboxyl esterase structurally related to the HSL family. Eur J Biochem. 2004;271:3953-3961.
18. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215:403-10.
19. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994;22(22):4673-4680.
20. Kelley LA, Sternberg MJE. Protein structure prediction on the web: a case study using the Phyre server. Nature Protocols. 2009;4:363-371
21. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory. Cold Spring Harbor, NY; 1989.
22. Sigurgisladottir S, Konraosdottir M, Jonsson A, Kristjansson JK, Matthiasson E. Lipase activity of thermophilic bacteria from Icelandic hot springs. Biotechnol Lett 1993;15(4):361-366.
23. Springer B, Master S, Sander P, Zahrt T, McFalone M, Song J, Papavinasasundaram KG, Colston MJ, Boettger E, Deretic V. Silencing of oxidative stress response in Mycobacterium tuberculosis:expression patterns of ahpC in virulent and avirulent strains and effect of ahpC inactivation. Infect Immun. 2001;69:5967–5973. [PubMed: 11553532].
24. Richter L, Saviola B. The lipF promoter of *Mycobacterium tuberculosis* is upregulated specifically by acidic pH but not by other stress conditions. Microbiol Res. 2009;164(2):228–232.
25. Geiman DE, Raghunand TR, Agarwal N, Bishai WR. Differential Gene Expression in Response to Exposure to Antimycobacterial Agents and Other Stress Conditions among Seven *Mycobacterium tuberculosis whiB*-Like Genes. Antimicrobial Agents And Chemotherapy, Aug. 2006;2836–2841
26. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using realtime quantitative PCR and the 2ΔΔC(T) Method. Methods 2001;25:402–408.

27. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, Gordon SV, Eiglmeier K, Gas S, Barry CE III, Tekaia F, Badcock K, Basham D, Brown D, Chillingworth T, Connor R, Davies R, Devlin K, Feltwell T, Gentles S, Hamlin N, Holroyd S, Hornsby T, Jagels K, Krogh A, McLean J, Moule S, Murphy L, Oliver K, Osborne J, Quail MA, Rajandream MA, Rogers J, Rutter S, Seeger K, Skelton J, Squares R, Squares S, Sulston JE, Taylor K, Whitehead S, Barrell BG. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nat (London) 1998;393:537-544.
28. Byun JS, Rhee JK, Kim ND, Yoon J, Kim DU, Koh E, Oh JW, Cho HS. Crystal structure of hyperthermophilic esterase EstE1 and the relationship between its dimerization and thermostability properties. Bmc Struct Biol. 2007;7:47.

    Available: http://www.pymol.org/.
29. Bloch H, Segal W. Biochemical differentiation of *Mycobacterium tuberculosis* grown *in vivo* and *in vitro*. J. Bacteriol. 1956;72:132–141
30. Cotes K, N'Goma JCB, Dhouib R, Douchet I, Maurin D, Carriere F, Canaan S. Lipolytic enzymes in *Mycobacterium tuberculosis*. Appl Microbiol Biotechnol 2008;78:741-749.
31. Cotes K, Dhouib R, Douchet I, Chahinian H, de Caro A, Carriere F, Canaan S. Characterization of an exported monoglyceride lipase from *Mycobacterium tuberculosis* possibly involved in the metabolism of host cell membrane lipids. Biochem J. 2007;408:417-427.
32. Deb C, Lee CM, Dubey VS, Daniel J, Abomoelak B. A novel *in vitro* multiple-stress dormancy model for *Mycobacterium tuberculosis* generates a lipid-loaded, drug-tolerant, dormant pathogen. PLoS One. 2009;4:e6077. doi:6010.1371/journal. pone.0006077.
33. Low KL, Rao PS, Shui G, Bendt AK, Pethe K, Dick T, Wenk MR. Triacylglycerol utilization is required for regrowth of *in vitro* hypoxic nonreplicating *Mycobacterium bovis* bacillus Calmette-Guerin. J Bacteriol. 2009;191:5037-5043.
34. Schue M, Maurin D, Dhouib R, N'Goma JCB, Delorme V, Lambeau G, Carriere F, Canaan S. Two cutinase-like proteins secreted by *Mycobacterium tuberculosis* show very different lipolytic activities reflecting their physiological function. FASEB J. 2010;24:1893-1903.
35. Vandal OH, Nathan CF, Ehrt S. Acid Resistance in *Mycobacterium tuberculosis.* J Bacteriol. 2009;191(15):4714–4721.
36. Jones CH. Case of Low Fever: Delirium: Incomplete Dementia: Convulsions: Death: Tuberculosis. Br Med J. 1867;344:84-85.
37. Lack NA, Kawamura A, Fullam E, Laurieri N, Beard S, Russell AJ, Evangelopoulos D, Westwood I, Sim E. Temperature stability of proteins essential for the intracellular survival of *Mycobacterium tuberculosis*. Biochemistry J. 2009;418:369-378.
38. Ganaie AA, Lella RK, Solanki R, Sharma C. Thermostable hexameric form of Eis (Rv2416c) protein of *M. tuberculosis* plays an important role for enhanced intracellular survival within macrophages. PLoS ONE 2011;6:e27590. doi:10.1371/ journal.pone.0027590.
39. Shen G, Singh K, Chandra D, Serveau-Avesque C, Maurin D, Canaan S, Singla R, Behera D, Laal S. LipC (Rv0220) is an immunogenic cell surface esterase of *Mycobacterium tuberculosis* Infect. Immun. 2012;80:243–253.

40. Bakala N'goma JC, Schue M, Carriere F, Geerlof A, Canaan S. Evidence for the cytotoxic effects of *Mycobacterium tuberculosis* phospholipase C towards macrophages. Biochim. Biophys. Acta. 2010;1801:1305–1313.
41. Singh G, Singh G, Jadeja D, Kaur J. Lipid hydrolizing enzymes in virulence: *Mycobacterium tuberculosis* as a model system. Crit Rev Microbiol. 2010;36(3):259-269.

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