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Characterization of LipN (Rv2970c) of *Mycobacterium tuberculosis* H37Rv and its Probable Role in Xenobiotic Degradation

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ABSTRACT

LipN (Rv2970c) belongs to the Lip family of *M. tuberculosis* H37Rv and is homologous to the human Hormone Sensitive Lipase. The enzyme demonstrated preference for short carbon chain substrates with optimal activity at 45°C/pH 8.0 and stability between pH 6.0–9.0. The specific activity of the enzyme was 217 U/mg protein with pNP-butyrate as substrate. It hydrolyzed tributyrin to di- and monobutyrin. The active-site residues of the enzyme were confirmed to be Ser216, Asp316, and His346. Tetrahydrolipstatin, RHC-80267 and N-bromosuccinimide inhibited LipN enzyme activity completely. Interestingly, Trp145, a non active-site residue, demonstrated functional role to retain enzyme activity. The enzyme was localized in cytosolic fraction of *M. tuberculosis* H37Rv. The enzyme was able to synthesize ester of butyric acid, methyl butyrate, in presence of methanol. LipN was able to hydrolyze 4-hydroxyphenylacetate to hydroquinone. The gene was not expressed in in-vitro growth conditions while the expression of *rv2970c* gene was observed post 6h of macrophage infection by *M. tuberculosis* H37Ra. Under individual in-vitro stress conditions, the gene was expressed during acidic stress condition only. These findings suggested that LipN is a cytosolic, acid inducible carboxylesterase with no positional specificity in demonstrating activity with short carbon chain substrates. It requires Trp145, a non active site residue, for it's enzyme activity. J. Cell. Biochem. 117: 390–401, 2016.

KEY WORDS: 4-HYDROXY PHENYLACETATE HYDROLASE; ESTERASE; LipN; *mycobacterium tuberculosis*; *rv2970c*; XENOBIOTIC

Tuberculosis is a deadly infectious disease caused by *M. tuberculosis* [Bloom and Murray, 1992]. From the genome sequence of *M. tuberculosis* H37Rv, a group of 24 genes were annotated as Lip family genes. Out of these, twelve proteins were found to be homologous to the human Hormone Sensitive Lipase (HSL) [Cole et al., 1998; Camus et al., 2002; Dedieu et al., 2013]. Amongst these, only LipY (Rv3097c) was identified as a lipase and has been reported to participate in the degradation of intra-or extracellular triacylglycerols (TAGs), thus releasing fatty acids as substrates to fuel the glyoxylate cycle [Deb et al., 2006; Mishra et al., 2008]. LipF (Rv3487c) and LipH (Rv1399c) were identified as cytosolic esterases, while LipC (Rv0220), a membrane bound/extracellular esterase interacted with host immune system to play an important role in infection and virulence [Zhang et al., 2005; Canaan et al., 2004; Shen et al., 2011]. In 2012, Delmore and colleagues showed that eight out of twelve *M. tuberculosis* Lip-HSL (mHSL) candidates were esterases and were inhibited by MmPPOX inhibitor. These Lip-HSL members might take part in metabolic processes and/or pathways involving short-chain substrates, such as signaling, membrane support, regulation and xenobiotic degradation [Delmore et al., 2012]. Recently the involvement of mycobacterium esterases in hydrolysis of xenobiotic esters for activation of prodrugs was also reported [Valente et al., 2011]. It suggested the importance of this metabolism in survival of a successful pathogen. The bioinformatic analysis using various tools revealed that many of these HSL like esterases could be involved in xenobiotic degradations.

Out of these 12 mHSL, LipN protein sequence demonstrated maximum identity with hHSL (38%). It contains all conserved motifs present in human HSL (Fig. S1). In KEGG orthology database, Rv2970c was predicted to be involved in xenobiotic degradation, particularly in bisphenol degradation pathway (KEGG pathway ID: mtu00363) (Fig. S2). An intermediate step of this pathway is hydrolysis of 4-hydroxyphenyl acetate (4-HPA) ester to hydroquinone (1.4-dihydroxybenzene). Bisphenol A degradation pathway has an arylesterase (EC 3.1.1.2) involved in conversion of 4-HPA to hydroquinone and acetate. We hypothesized that if LipN was

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involved in bisphenol A degradation then that arylesterase (EC 3.1.1.2) could possibly be LipN (Fig. S3). This hypothesis was based on the fact that LipN demonstrated high similarity with HapB 4-hydroxyphenyl acetate hydrolase of *P. fluorescens* involved in hydrolysis of 4-HPA to hydroquinone [Kamerbeek et al., 2001, 2003a, 2003b].

Therefore, in the present study an attempt has been made to elucidate the biological activity of Lip N as a 4-HPA hydrolase and in ester hydrolysis/synthesis. The enzyme was characterized in detail. The expression pattern of the gene in *ex vivo* and in vitro conditions (normal growth and in vitro stress conditions) might give insight to it's importance during stress conditions.

MATERIALS AND METHODS

MATERIALS

All the synthetic substrates (pNP-esters), RPMI-1640, fetal bovine serum (FBS), Phorbol 12-myristate 13- acetate, (PMA) and Tri reagent were purchased from Sigma Chemical Co, St. Louis, MO. Isopropyl D-thiogalactopyranoside (IPTG) was purchased from Golden Biotech (GBT), Taiwan. Ni–NTA His binding resin was purchased from Qiagen, Germany. Ampicillin, kanamycin, rifampicin, LB media, Middlebrook 7H9 broth base, Urea, Lipase from *Aspergillus niger* were purchased from Himedia, India. OADC supplement was purchased from BBL(BD bioscience, Fraklin Lakes, NJ), Analytical standard 4-HPA and hydroquinone were purchased from TCI Japan and Sigma Chemical Co., respectively. pQE30 UA vector was purchased from Invitrogen. All primers used in study are given in Table I.

METHODS

Growth of *M. tuberculosis* H37Ra and THP-1 cell line. *M. tuberculosis* H37Ra was procured from Microbial Type Culture Collection and Gene Bank (MTCC) Chandigarh, India. It was grown in middlebrook 7H9 broth base supplemented with 1% glycerol and 0.05% Tween-80. An additional 10% (v/v) growth supplement OADC was added. The human leukemic monocytic cell line THP-1 was obtained from the NCCS Pune, India. The cells were cultured in 10 cm tissue culture dishes in RPMI medium supplemented with 10% charcoal-treated FBS, 2% non-essential amino acids and 50 mg/ml penicillin/streptomycin at 37°C in 5% CO_2.

Infection of THP-1 macrophages with *Mycobacterium tuberculosis* H37Ra. *Mycobacterium tuberculosis* H37Ra was grown to log phase in small volumes of 7H9 broth supplemented with 10% (v/v) OADC (BBL). Prior to infection, *M. tuberculosis* cells were washed three times with sterile phosphate buffer saline, pH 7.4 (PBS) dispersed by vortexing followed by sonication (15 s, 500 W) in a sonicator [Tanigawa et al., 2008] and then passed through 30-gauge needle several times to get single cell-suspension. For infection experiments, the THP-1 cells were procured from the ATCC and maintained in RPMI-1640 supplemented with 10% FBS, 1% HEPES, 1% L-glutamine, 100 μg/ml streptomycin, 100 IU/ml penicillin. The cells were maintained at 37°C in humidified atmosphere of 5% CO_2. Media was changed after every 3–4 days. THP-1 cells were plated in 6 well flat bottom plates at 3×10^5 cells per well. After 24 h cells were incubated with fresh media with 10 μg/ml PMA for differentiation. After 48 h PMA treatment, cells were washed with PBS and incubated with fresh RPMI media for 8 h. Later the cells were infected with diluted and dispersed preparations of *M. tuberculosis* at multiplicity of infection (MOI) of 20:1 (20 bacilli per macrophage). After 4 h, the cells were washed four times with PBS to remove extracellular bacteria following treatment with gentamycin (200 μg/ml) for 4 h and then incubated again with fresh RPMI-1640 medium supplemented with 10% fetal calf serum L-glutamine (2 mM), HEPES (2.38 mg/ml), sodium bicarbonate (2.2 mg/ml), 2-mercaptoethanol (0.05 mM) and gentamycin (20 μg/ml). Bacteria were isolated from macrophage at specific time points (0 h, 6 h) after treatment of infected cells with 0.1% Triton X 100 in PBS followed by RNA isolation. Cells initially incubated with mycobacterium for 4 h were taken as 0 h sample.

***rv2970c* expression analysis under different stress conditions.** Sequence analysis of *rv2970c* gene and 1000 bp upstream region showed 100% identity in *M. tuberculosis* H37Ra and *M. tuberculosis* H37Rv. Also *rv2970c* was not predicted to be regulated by Phop regulon reported to be responsible for attenuation of *M. tuberculosis*

TABLE I. Primers Used in Entire Study

Primer for cloning Name of gene	Forward primer	Reverse primer
rv2970c	5′ATGACCAAAGAGTCTGCCAGG 3′	5′TCAAACCCGGCTAAGGTG 3′
Primers used in Stress induced expression study **Name of genes**	**Forward primer**	**Reverse primer**
rv2970c	5′CGACGTGACCGACCTGTCAT 3′	5′TGGGCCCACCCTCGTAGC 3′
16S rRNA (semi quantitative RT PCR)	5′AGAGTGTGATCCTGGCTCAG 3′	5′TACGGYTACCTTGTTACGACTT3′
16S rRNA (real time)	5′GAGGAAGGTGGGGATGACGT 3′	5′AGGCCCGGGAACGTATTCAC 3′
Primers used for site directed mutagenesis **Position where point mutation created**	**Forward primer**	**Reverse primer**
S216A	5′TGGGCGGGGACGCTGCGGGCGGCAACCTGT 3′	5′ACAGGTTGCCGCCCGCAGCGTCCCCGCCCA 3′
D316A	5′ TCGCCGGCTTCGCCCCATTGCGCGAC 3′	5′ GTCGCGCAATGGGGCGAAGCCGGCGA 3′
D320A	5′TCGACCCATTGCGCGCCGAAGGAGAAAGC 3′	5′GCTTTCTCCTTCGGCGCGCAATGGGTCGA 3′
H346A	5′ TGGGTTCGCTGACGGCTGGCTTCCTCAACC 3′	5′ GGTTGAGGAAGCCAGCCGTCAGCGAACCCA 3′
W145A	5′ CACGGTGGCGGCGCGACGCTCGGGGAC 3′	5′GTCCCCGAGCGTCGCGCCGCCACCGTG 3′

H37Ra strain [Gonzalo Asensio et al., 2006]. Many studies reporting gene expression profile difference between *Mycobacterium tuberculosis* H37Rv and *Mycobacterium tuberculosis* H37Ra were taken in to consideration [Zheng et al., 2008; Gao et al., 2004]. To the best of our knowledge there was not a single report suggesting difference in expression of *rv2970c* in *Mycobacterium tuberculosis* H37Rv and *Mycobacterium tuberculosis* H37Ra. Therefore we used *M. tuberculosis* H37Ra for expression studies.

The bacterial cells were grown to mid log phase (A_{600} 0.7–0.8) and harvested by centrifugation. The bacterial pellet was washed with PBS and resuspended in different media for exposure to stress conditions such as oxidative stress (7H9 growth media with 5 mM H_2O_2) [Springer et al., 2001], acidic stress (7H9 growth media at pH 4.5 (adjusted with 0.2 N HCl) [Richter and Saviola, 2009] and nutrient stress (1X phosphate buffer saline) [Geiman et al., 2006]. These three stressed cultures were incubated for 6 h, alongwith control culture in middlebrook 7H9 broth base (normal pH, without any additive).

RNA samples of *Mycobacterium tuberculosis* H37Ra was isolated using TRI reagent as per company's instruction manual. The integrity, quantity and quality of RNA samples were checked by agarose gel electrophoresis and spectrophotometry. All the RNA samples were given Ambion® TURBO DNA-free™ DNase Treatment as per company's instruction manual before being converted into complementary DNA (cDNA) using RevertAid™cDNA kit (Fermentas). The cDNA was used as a template to check the relative expression of the *rv2970c* gene. 16S-rRNA was used as an internal control to normalize the expression and then it was normalized to the control/untreated condition for the target gene. cDNA was diluted to half for the expression work of 16S rRNA. a) Semi quantitative PCR: It was performed with cDNA corresponding to 50 ng RNA using Fermentas Taq polymerase. Separate sets of reaction master mix were prepared by adding the following components for a 25 μl reaction each: 1 μl each of forward and reverse primers from 10 μM solution, 1 μl cDNA, 2.5 μl of 10X enzyme buffer, 1.5 μl of 25 mM $MgCl_2$, 1 μl of 25 mM dNTPs, 0.5 μl Taq enzyme (5 U/μl) and rest double distilled water to complete 25 μl reaction mix. The products of RT PCR were checked on 1.2% agarose gel. b) Real time PCR-analysis: Real time PCR was performed with cDNA corresponding to 50 ng RNA using DyNAmo™ ColorFlash SYBR® Green qPCR Kit from Thermo-scientific in Mastercycler ep realplex® Real-Time PCR System (Eppendorf). Reaction mix (20 μl) was prepared by adding the following components: 10 μl of master mix (containing enzyme, 1 mM dNTPs, enzyme buffer, 2.5 mM $MgCl_2$ and SYBR Green dye), 0.4 μl of 25 μM ROX dye, 1 μl each of forward and reverse primer and double distilled water to make up the volume. In case of mycobacterium infected cell line, relative expression values of *rv2970c* were calculated w.r.t expression value of 16S rRNA, taken as 1. For relative expression of LipN in different stresses w.r.t control, determination of fold change in expression level of the gene was carried out by using the $2^{-\Delta\Delta Ct}$ Method [Livak and Schmittgen, 2001].

Cloning, expression, and purification of LipN. *rv2970c* ORF was amplified by standard PCR procedure from *M. tuberculosis* H37Rv genomic DNA (kind gift from Dr. U.D Gupta). Primers used were as described in Table I. Amplified gene was cloned in pQE30 UA vector and it was used to transform *E. coli* M15 cells. Transformed *E. coli* M15 colonies with correct ORF were confirmed by colony PCR followed by sequencing of the positive clones Overnight grown culture with cloned gene was used to inoculate (1%) 250 ml Luria Bertanii (LB) media containing 30 μg/ml kanamycin and 100 μg/ml ampicillin. The cultures were grown till OD_{600} reached ~0.5–0.6 and recombinant protein expression was induced by addition of 0.1 mM IPTG. Cells were incubated at 37°C, harvested after 3 h of induction and the pellet was resuspended in lysis buffer (50 mM Tris-HCl pH-8.0, 50 mM NaCl, 1 mM EDTA, 0.5% Triton-X, 0.25 mg/ml lysozyme) and stored at −80°C. Cells were disrupted by ultrasonication (10× with 15 s cycle) followed by centrifugation. LipN protein was exclusively present in inclusion bodies. Inclusion body pellet was dissolved in solubilizing buffer (100 mM NaH_2PO_4 buffer pH 8.0, 10 mM Tris/HCl buffer pH 8.0, 8 M Urea) and kept on a rocker for 30 min at room temperature. Cell debris was removed by centrifugation at 10,000 rpm for 10 min. Supernatant was loaded on previously equilibrated Ni^+-NTA affinity column with buffer (100 mM NaH_2PO_4 buffer pH 8.0, 10 mM Tris/HCl buffer pH 8.0, 8.0 M Urea). Washing of unbound proteins and elution of protein was in pH dependent manner. The column was washed with 10 column volume washing buffer (100 mM NaH_2PO_4 buffer pH 6.8, 10 mM Tris/HCl buffer pH 6.8, 8 M Urea), and the protein was eluted with elution buffer (100 mM NaH_2PO_4 buffer pH 5.8, 10 mM Tris/HCl buffer pH 5.8, 8 M Urea). Eluted fractions containing purified LipN were analyzed by denaturing polyacrylamide gel electrophoresis (SDS PAGE).

For refolding of LipN, step wise dialysis was carried out with decreasing concentration of urea ranging from 6.0 to 1.0 M and at last in buffer (50 mM NaH_2PO_4 buffer pH 8.0) for overnight at 4°C. Protein concentration was calculated after purification of LipN and before dialysis. It's concentration was considered as a 100% protein. After dialysis insoluble aggregates (misfolded /unfolded proteins) were removed by centrifugation and soluble aggregates were removed by 50 kd cut-off concentrator. Remaining protein was considered as a refolded protein and its concentration was calculated by Lowry's method. This concentration was considered as % refolded protein. Nearly 35% of protein was refolded through this procedure. Protein was concentrated to final concentration of 1 mg/ml of protein. Enzyme activity of the protein was determined.

Standard enzyme assay. Enzyme activity was determined by the modified method of Dosanjh and Kaur [2002]. To 750 μL of 50 mM NaH_2PO_4 buffer pH 8.0, 100 μL of pNP-butyrate/substrate (2mM, dissolved in ethanol), 100 μl of 10 mM sodium deoxycholate and 50 μl of enzyme was added and mixed. Reaction mixture was incubated at 45°C for 10 min followed by addition of 250 μl of 0.1 M Na_2CO_3. Enzyme activity was determined by measuring absorption at A_{420} and specific enzyme activity was calculated. One unit of enzyme activity is defined as the amount of enzyme, which librates 1 μ mole of pNP from pNP-ester per minute under standard assay conditions.

Sub-cellular localization of LipN in *M. tuberculosis* H37Ra. Rabbits were bled before immunization. Thick emulsion of LipN (0.50 mg) mixed with Freund's adjuvant (was injected subcutaneously to immunize two rabbits (Approved by Institutional animals ethics committee/ IAEC/127) on day 0. Three booster doses were given at the intervals of 15 days with emulsified LipN (0.25 mg) in

incomplete Freund's adjuvant. Five days after last booster dose, blood was collected and serum was isolated. Immunoglobin G (IgG) was purified from the immunized rabbit's serum by A-Sepharose 4B columns (Amersham Biosciences). Presence of antibodies in serum was checked by Ouchterlony double immunodiffusion and used for western blot analysis.

The culture of acid stressed *M. tuberculosis* H37Ra (at midlog phase, A_{600}–0.8) was used to isolate various sub-cellular functions as described in Rezwana et al. [2007]. For western blot analysis, equivalent amount of each fraction were loaded on 12% SDS-PAGE along with purified recombinant LipN and transferred to PVDF membrane. After blocking the PVDF membrane with 5% skimmed milk in TBS, the PVDF membrane was incubated with polyclonal antibodies against LipN (final dilution 1:500) for 2 h. After three washes with tris buffer saline (50 mM Tris-Cl, pH 7.5, 150 mM NaCl TBS), the membrane was incubated with HRP-tagged anti rabbit secondary antibodies for 2 h at room temperature, (final dilution 1:10000). The blot was thoroughly washed with TBS and developed by developing solution (TAB, Tri ammonium benzoate along with H_2O_2) (Fermentas).

BIOCHEMICAL PROPERTIES

Substrate specificity. To investigate the substrate specificity of LipN, the enzyme activity was measured with various substrates like pNP-acetate (C-2), pNPbutyrate (C-4), pNP-octonate (C-8), pNP-decanoate (C-10), pNP-laurate (C-12), pNP-myristate (C-14), pNP-palmitate (C-16), pNP-stearate(C-18). Standard enzyme assay was performed as described above. Emulsified tributyrin and triolein were also used to check its degradation by LipN on substrate plates by well assay method.

Thin layer chromatography. Tributyrin, emulsified in 50 mM phosphate buffer (1% V/V), was incubated with 50 μl of enzyme (40 U/ml) in 1 ml of reaction mix for 16 h. The samples were taken out at different time interval. Lipids were extracted with 2:1 chloroform: methanol followed by gentle mixing and centrifugation at 10000 rpm for 5 min. Four microliters of extracted products were run on silica coated TLC plate along with tributyrin, dibutyrin and monobutyrin as a control. Chloroform: acetic acid (95:5) was used as a mobile phase and iodine fumes were used to visualize the products on TLC.

Effect of pH and temperature on enzyme activity and stability. To find out pH optima for esterase activity, enzyme assay was performed in buffers (50 mM) of different pH from pH 4.0–10.0. Acetate buffer (pH 4-5) phosphate buffer (pH 6.0–8.0), Tris-HCl buffer (pH 8.0–9.0) and carbonate-bicarbonate buffer (pH 11.0) were used. To determine optimum temperature of enzyme activity, enzyme assay was performed at various temperatures (25–75°C) for 10 min. Enzyme activity was determined as per standard enzyme assay protocol.

For pH stability 50 μl of enzyme was incubated with 100 μl of buffers at different pH (pH 4.0–10.0) for 30 min at 25°C. Afterwards enzyme assay was performed at optimum pH and temperature. To obtain temperature stability profile, 50 μl of enzyme was incubated at various temperatures (25–70°C) for 30 min followed by incubation at 4°C for 20 min. Enzyme assay was performed as per standard assay conditions.

Enzyme kinetics. Enzyme activity of the purified LipN was determined as a function of substrate concentration (0.01–1.0 mM of pNP-butyrate) using standard assay method. The Michaelis–Menten constant (K_m), maximum velocity for the reaction (V_{max}) and catalytic constant (k_{cat}) were calculated.

Time dependent reaction progress curve of hydrolysis of pNP-butyrate. Effect of time on conversion of substrate into product by LipN and lid domain containing lipase from *Aspergillus niger* were analyzed in a time dependent manner. The enzymes were incubated with pNP-butyrate (200 μM) at their optimum temperatures i.e. 45°C for LipN and 37°C for lipase from *Aspergillus niger* in different vials. The vials were taken out after every min of incubation and absorption was measured at A_{420}.

Effect of different additives and effectors on enzyme activity. The effect of various reagents on LipN enzyme activity was determined. Purified LipN protein was incubated for 1 h at 25°C with reagents (5 mM) such as N-Bromosuccinimide (NBS, oxidizes tryptophan and transforms it into an oxindole residue), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC, modifies/inactivates acidic amino acid residues), Iodoacetae (IA, cysteine alklytaing agent), citraconic anhydride (CA, lysine modifier), phenylglyoxal monohydrate (PG, phospholipase A_2 inhibitor), phenylmethanesulfonylflouride (PMSF, Serine modifier) and Diethylpyrocarbonate (DEPC, Histidine modifier), N-acetyl imidazole (NAI, tyrosine modifier), RHC 80267 and tetrahydrolipstatin (THL), a known DAG and TAG lipase/esterase inhibitor respectively [Sutherland and Amin, 1982; Yang et al., 2010]. After incubation the relative enzyme activity was determined by standard assay method. The enzyme without any additive was taken as control (100% enzyme activity).

Site directed mutagenesis. Site directed mutagenesis was carried out by substituting conserved putative active site residues Ser216, Asp316, Asp320 and His346 with alanine by using site directed mutagenesis kit (Stratagene, Santa Clara, CA). A conserved tryptophan (Trp145) in the vicinity of active site was also mutated to alanine. The primers are listed in Table I. *E. coli* M15 cells were transformed with plasmids having confirmed mutant gene. Mutant proteins were purified, checked for enzymatic activity and compared with the wild type enzyme.

Esterification of butyric acid by immobilized LipN. Purified LipN was immobilized on HP 20 beads (Diaion) as described by Dosanjh and Kaur [2002]. Thermal inactivation of aqueous and immobilized enzyme was studied by incubating enzyme at 60°C for different time points with an interval of 10 minutes each and assayed for enzyme activity by standard assay method. Both type of enzymes without any pretreatment were taken as control (100%). HP20 beads (200 mg) with immobilized LipN enzyme were used as biocatalyst for the esterification of butyric acid (0.25 M) and methanol (0.4 M) in hexane in 10 ml reaction mix for different time points in duplicate at 37°C with heat inactivated enzyme as a control. To the 10 ml reaction mixture, 20 ml acetone: methanol 1:1(v/v) was added. The ester content was quantified by using alkalimetric method of titrating unreacted acid with 0.1 N NaOH with phenolphthalein as an indicator. The conversion (%) in ester synthesis was determined based on acid consumed [Bovara et al., 1993].

LC MS analysis. 4-hydroxyphenyl acetate hydrolase activity of LipN was checked by using 4-HPA as substrate by LC-MS. Two hundred microliter of 4-HPA (45 mM) prepared in ethanol was added to 800 μl of 50 mM phosphate buffer with HP20 immobilized LipN

(40 U/20 mg of HP20 beads) and incubated for 2 h at 45°C on shaker in dark followed by LCMS analysis. One ml of 4-HPA and hydroquinone (10 mM) in 50 mM phosphate buffer without enzyme were incubated for 2 h at 45°C on shaker and were taken as a standard for LC MS experiment. Waters Micromass Q-TOF Micro LCMS available at Sophisticated Analytical Instrumentation Facility (SAIF), CIL and UCIM at Panjab University was used for analysis. For liquid chromatography separation was achieved by using 5 μ Supelco LC-18-DB, 2.1 × 100 mm column. Mobile phase (0.1:25:75 phosphoric acid: methanol: water) in 100% isocratic mode with flow rate of 0.15 ml/min was used. Ten microliter sample was injected. Hydroquinone and 4-HPA were detected at 219 nm and 280 nm respectively. For TOF MS (ES-) negative ionization mode, analysis was performed in the negative atmospheric pressure. Chemical ionization mode was attained using a vaporizer temperature of 450° C and a capillary temperature of 150°C. Nitrogen was used as a sheath gas (20%) and auxiliary gas (5%). Capillary voltage was 2823 V. and sample cone for fragmentation was 30 V.

RESULTS

BIOINFORMATICS ANALYSIS

Bioinformatics analysis revealed that LipN belongs to bacterial family IV carboxylesterases (Fig. S1) and shows sequence similarity to the human HSL [Arpigny and Jaeger, 1999] which is produced during nutritional stress i.e. fasting in presence of epinephrine [Lampidonis et al., 2011]. All four conserved sequences of HSL (consensus pentapetide GDSAG motif which contains active site serine residue, conserved HGGG/GGGX motif containing oxyanion region and two C-terminus located conserved motifs, DPLR and HGF) were present in LipN (Fig. S1). Protein BLAST result with PDB showed LipN to be 41% identical to thermophilic carboxylesterase Est2 from *Alicyclobacillus acidocaldarius* with 83% query coverage and 39% identical with HSL-like carboxylesterase from *Sulfolobus tokodaii* with query coverage 78% (data not shown).

rv2970c GENE EXPRESSION ANALYSIS

No expression of *rv2970c* gene was observed in mid log phase of in-vitro grown culture of *M. tuberculosis H37Ra*. In THP-1 cells infected with *M. tuberculosis H37Ra*, the gene was expressed after 6 h of infection (Fig. 1A). The mycobacterium is exposed to several stress conditions inside the macrophages. Therefore expression pattern of *rv2970c* was studied in individual stress conditions such as oxidative, acidic and nutritive stress. Semi quantitative PCR analysis demonstrated that the *rv2970c* gene was expressed only in acidic stress condition (Fig. 1B). No expression was observed in control oxidative and nutritive stress conditions. The normalized values of expression (*rv2970c/16S RNA*) as demonstrated by real time PCR showed 22 fold higher expression of *rv2970c* in acidic condition when compared to control (Fig. 1C).

PURIFICATION, REFOLDING, AND CELLULAR LOCALIZATION OF LipN

Attempts to express recombinant LipN protein in soluble fraction at different induction time and temperatures as well as concentrations of IPTG were unsuccessful. Consequently, protocol to isolate recombinant protein from inclusion bodies was standardized. The denatured protein was purified to homogeneity (Fig. 2A). After step wise dialysis, the misfolded/unfolded proteins were removed by centrifugation. Nearly 35% of total protein was refolded to active protein. Purified protein was of 42 kDa as observed in SDS-PAGE. The size of protein was little higher due to the presence of His tag encoded by vector sequence. The specific activity for *rv2970c* gene product was 217 U/mg with pNP butyrate as substrate. Anti-LipN polyclonal antibodies were raised in rabbits and were used to determine the sub cellular distribution of LipN in *M. tuberculosis*. The antibody titer for LipN was low (data not given). Therefore purified IgG was used for western blot analysis. The polyclonal antibodies recognized both, purified as well as native LipN present in the cytosolic fraction of acid induced *M. tuberculosis* H37Ra (Fig. 2B).

BIOCHEMICAL CHARACTERIZATION OF LipN

Amongst the pNP substrates, LipN protein showed preference for short carbon chain substrates. Though the enzyme showed highest activity with pNP-acetate, we chose to use pNP- butyrate as substrate because of the self hydrolysis of pNP-acetate. Preference over wide range of substrates having smaller acyl chain was evident in the results (Fig. 3A). It showed a zone of clearance on emulsified tributyrin plate but no activity on emulsified trioline plate (data not shown). LipN hydrolyzed tributyrin to dibutyrin and monobutyrin (Fig. 3B).

The optimum enzyme activity was observed at pH 8.0 and temperature 45°C (Figs. 3C, D). The enzyme was stable in the range from pH 6.0 to pH 9.0 (Fig. 3 D). About 80% of enzyme activity was retained after incubation of protein at 45°C for 30 min (Fig. 3C). The apparent K_m and V_{max} were found to be 303 μM and 75.75 μmol min^{-1} respectively. The apparent k_{cat} and k_{cat}/K_m for LipN were determined as 7283 min^{-1} and 24.03 $\mu M^{-1} min^{-1}$ respectively. The time course of reaction progress of hydrolysis of pNP-butyrate by LipN showed progressive increase in hydrolytic rate, a steady state reaction did not reach saturation in 8 min. It did not show latency phase as observed in lid containing lipase from *Aspergillus niger* (Fig. 4A,B). To investigate the role of conserved amino acid in structural and functional attributes of LipN, effect of various amino acid modifiers were investigated. NBS, THL and RHC, PMSF and DEPC inhibited LipN enzyme activity completely whereas citraconic anhydride, acetyl imidazol, phenylglyoxal, iodoacetate EDAC showed no effect on enzyme activity (Table II).

Tryptophan 145 and Active Site Residues are Indispensible for Esterase Activity of LipN

Bioinformatics study illustrates LipN as a protein with conserved pentapeptide GDSAG (G-X-S-X-G), a characteristic of α/β hydrolases, spanning between 214 to 218 amino acids. Other conserved active site residues included, Asp316, Asp320 and His346. The individual single substitution mutations for predicted active site residues Ser216, His346 and Asp316 to Ala led the loss in the enzyme activity of LipN while Asp320 substitution did not affect the enzyme activity (Fig. 4C). Trp145, another conserved residue, was mutated due to it′s proximity and geometry with active site residues observed through structure modeling (Fig. S5). On substituting Trp145 to Ala, this mutant protein also lost enzymatic activity completely (Fig. 4C).

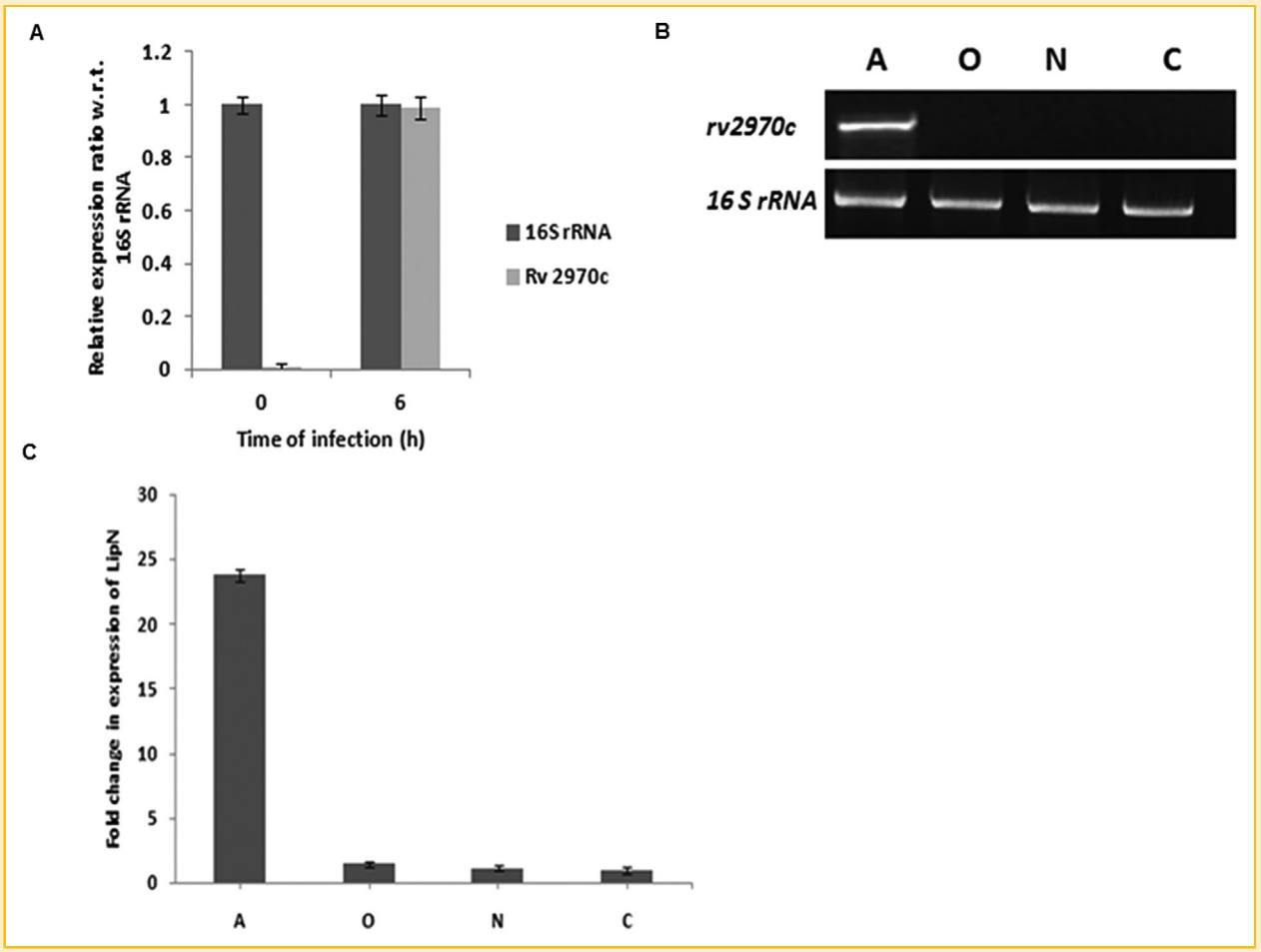

Fig. 1. Ex-vivo and in-vitro stress induced expression of *rv2970c* A: Expression of *rv2970c* after infecting macrophages (THP-1) with *M. tuberculosis* H37Ra. The samples were collected after 6 h. B: Semi quantitative PCR based *rv2970c* mRNA expression under various stress conditions. The mycobacterium culture in mid log phase was exposed to acidic (A), oxidative (O), nutritive (N) along with control (C) for 6 h. C: Real time PCR based *Rv2970c* (LipN) mRNA expression.

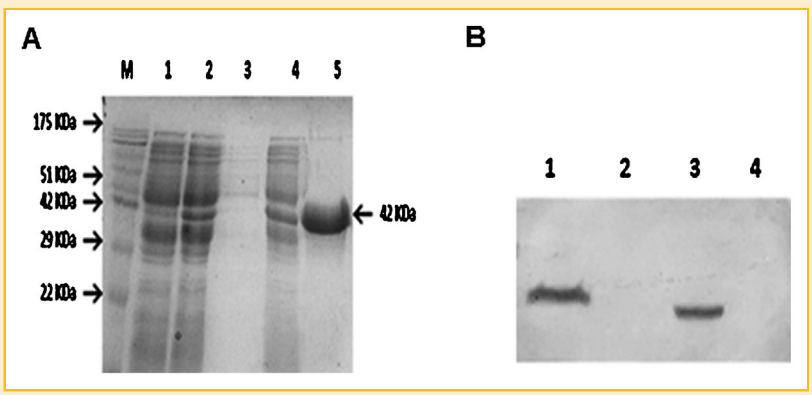

Fig. 2. Expression and Purification of LipN (Rv2970c) A: SDS-PAGE analysis: Lane M: Protein MW Marker, 1: Uninduced LipN, 2: IPTG induced LipN culture pellet. 3: lysate after sonication of induced LipN sample. 4: Induced LipN pellet/ inclusion bodies 5: Purified LipN. B: Subcellular localization of LipN in acid induced Mycobacterial cell fractions. Polyclonal antibodies were raised in rabbits against LipN. lane 1: Purified recombinant LipN, Lane 2: Culture filtrate protein fraction, Lane 3: Cytosolic protein fraction, Lane 4: Cell wall fraction.

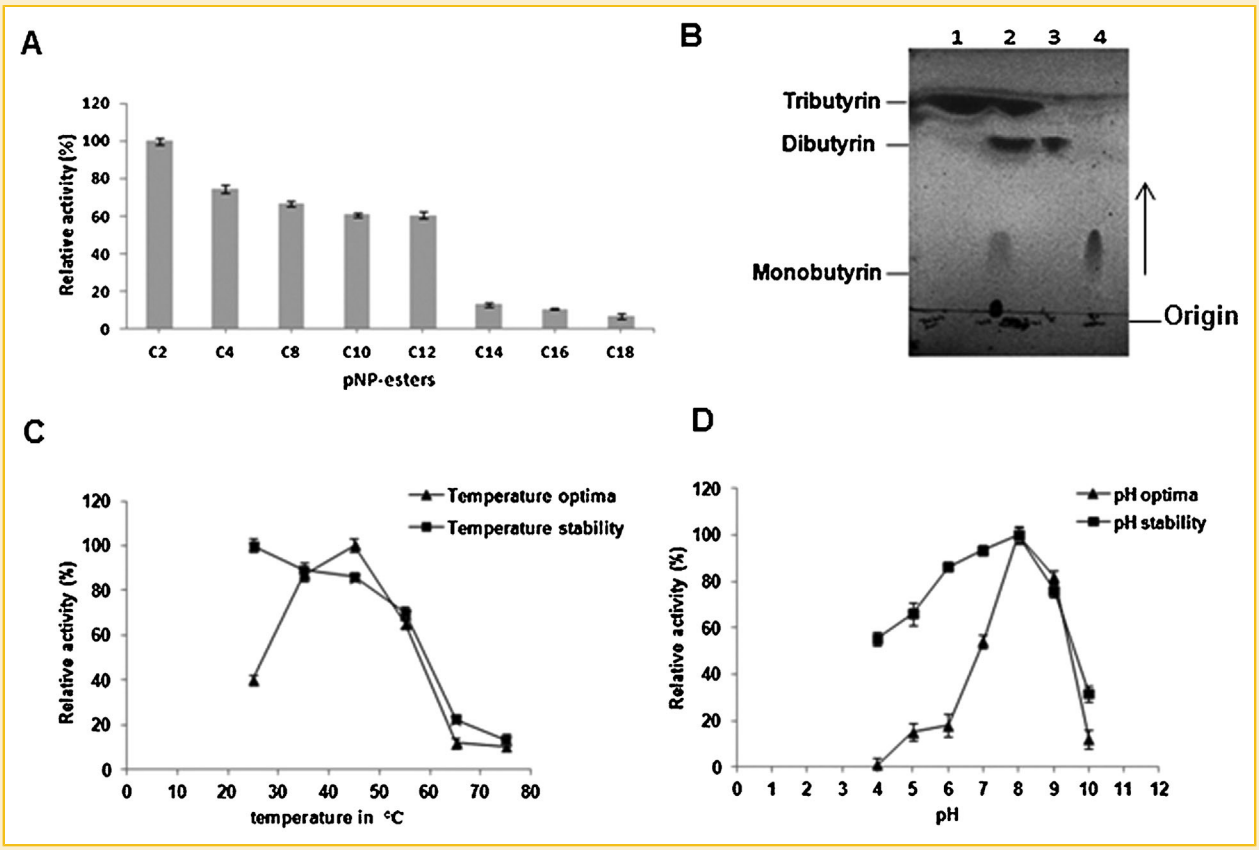

Fig. 3. Studies on biochemical characteristics of LipN. A: Substrate specificity of LipN with different pNP esters by standard enzyme assay as described previously: where, C2- pNP-acetate, C4- pNP-butyrate, C8- pNP-caprylate, C10- pNP-capricate, C12- pNP-laurate, C14- pNP- myristate, C16- pNP-palmitate, C18- pNP- sterate. B: Analysis of tributyrin hydrolysis by LipN on thin layer chromatography. 1: Tributyrin 2: Tributyrin and its hydrolyzed products after incubating with LipN. 3: Dibutyrin 4: Monobutyrin. C: Effect of temperature on enzyme activity and stability of LipN. D: Effect of pH on enzyme activity and stability of LipN. (Each experiment was conducted independently three times in triplicate. Error bars indicate the standard deviation).

IMMOBILIZED LipN SYNTHESIZES METHYL BUTYRATE THROUGH ESTERIFICATION

Activity of the enzyme immobilized on HP20 beads was determined to be 2U+-0.4/mg of beads. Immobilization increased thermal stability of enzyme activity to 60°C. The half life of aqueous and immobilized LipN was nearly 5 min and 35 min respectively at 60°C (Fig. 5A). Esterification studies of enzyme illustrated that 50% of butyric acids present in the reaction mix were converted to methyl butyrate in the presence of methanol after 12 h incubation with LipN enzyme. After 16 h incubation 58% esters were produced (Fig. 5B).

LC MS ANALYSIS OF 4-HYDROXYPHENYL ACETATE HYDROLASE ACTIVITY OF LipN

The conversion of 4-HPA to hydroquinone by LipN was studied (Fig. 6A). An analytical standard 10 mM 4-HPA peak was detected at 280 nm with retention time 2.10 min (Fig. 6B) and standard 10 mM hydroquinone was detected at 219 nm with retention time 3.43 min (Fig. 6C). However, upon oxidation it might have been converted to p-benzoquinone which was detected with retention time 2.08 (Fig. 6C). In test sample after incubation of 4-HPA with LipN for 2 h, both hydroquinone, substrate 4-HPA and p-benzoquinone were detected with same retention time (Fig. 6D). In TOF MS (ES-), analytical standard 4-HPA base peak was detected at 151 m/z with ion intensity 9446 and fragments of 4-HPA (unknown chemical formula) were m/z 107 and 106 (Fig. 7A). However, fragment pattern of 4-HPA was same as observed in HMDB (http://www.hmdb.ca/spectra/ms_ms/2558) (http://www.hmdb.ca/spectra/ms_ms/33). Analytical standard hydroquinone base peak was detected at 109 m/z with 2487 ion intensity (Fig.7B) and fragment pattern was as observed earlier (http://foodb.ca/spectra/ei_ms/338). In case of test sample both 4-HPA and hydroquinone peaks were detected at m/z 151 and m/z 109, respectively. A significant 4-HPA hydrolysis was detected (Fig. 7C)

DISCUSSION

For better treatment of TB, it is prerequisite to understand the growth conditions and physiological environments experienced by bacteria inside the host. Under stress conditions, *M. tuberculosis* shifts to alternative carbon sources for it's persistence. Many microbes use 4-hydroxyphenylacetate as a sole carbon source. 4-HPA is a common catabolic product of L-tyrosine, aromatic biogenic amine and

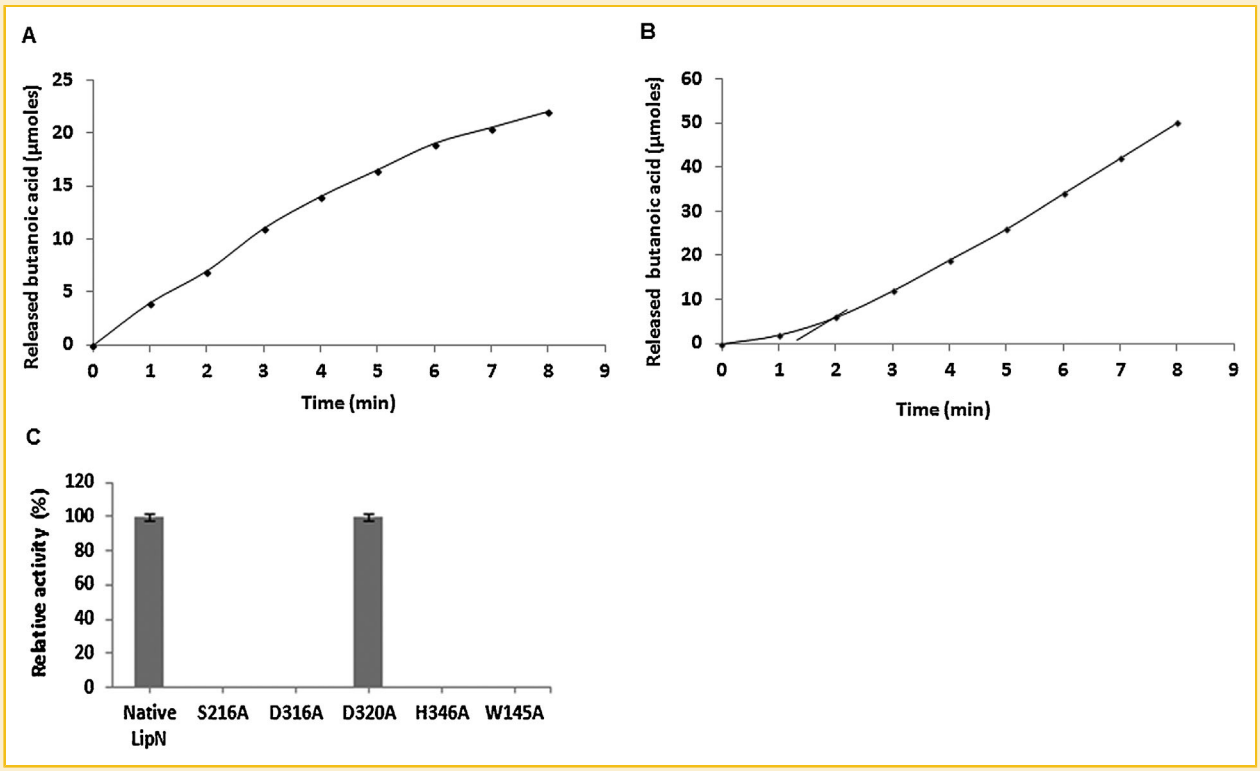

Fig. 4. Time dependent kinetics of LipN and Active site residue determination. A: The time dependent reaction progress curve of hydrolysis of pNP-butyrate (200 µM) by the LipN. B: The time dependent reaction progress curve of hydrolysis of pNP-butyrate (200 µM) by the Lipase from *Aspergillus niger* (Lid domain containing true lipase) enzyme. Latency period for Lipase was calculated from the intercept between asymptote of the progress curve and X axis which is 1.2 min. C: Relative enzyme activity of various mutants (Ser216Ala, Asp316Ala, Asp320Ala, His346Ala, Trp145Ala) in comparison to native LipN protein. (Each experiment was conducted independently three times in triplicate. Error bars indicate the standard deviation).

phenolic compounds like 4-hydoxyacetophenone and bisphenol degradation [Díaz et al., 2001].

As per our hypothesis, through LC-MS TOF analysis, we could identify the hydrolysis of 4-HPA to hydroquinone in the presence of LipN which might be acting as an arylesterase (EC 3.1.1.2). Though almost same retention time of 4-HPA and hydroquinone was observed in LC for reason unknown, hydroquinone formation was clearly evident in MS TOF analysis. Promoter analysis of LipN gene revealed Rv0047c binding site located 267 bp upstream of its start codon (http://genome.tbdb.org/cgi-bin/get_binding.cgi). Rv0047c has conserved domain of PadR transcription regulator, (a repressor of phenolic acid stress response) from HTH-XRE (helix turn helix-xenobiotic response element) super family which regulates the xenobiotic response. Also LipN was predicted to have strong functional association with 7 Cytochrome p450 proteins suggesting it's role in xenobiotic degradation (STRING 9.1, Fig. S4). The enzymes metabolizing xenobiotics are pharmaceutically important as these might be responsible for the breakdown of medications also.

Carboxylic ester hydrolases (EC 3.1.1._) are a diverse group of enzymes that hydrolyze carboxylic esters. Since LipN hydrolyzed tributyrin it can be designated as a carboxylesterases (EC 3.1.1.1) too.

TABLE II. Effect of Additives and Effectors on Enzyme Activity

Modifier/additives/effecters	Concentration	Relative enzyme activity (in %)
Control (no treatment)	–	100
Tetrahydrolipstatin (THL)	5 mM	Not detectable
RHC -80267	5 mM	Not detectable
N- Bromosuccinimide	5 mM	Not detectable
Iodoacetic acid	5 mM	100 ± 6
Acetyl imidazole	5 mM	100 ± 4
1-ethyl-3-(3- dimethylaminopropyl) carbodiimide (EDAC)	5 mM	100 ± 6
Phenylglyoxal monohydrate	5 mM	100 ± 2
Citraconic anhydride	5 mM	100 ± 2
PMSF	10 mM	10 ± 6
DEPC	10 mM	18 ± 2

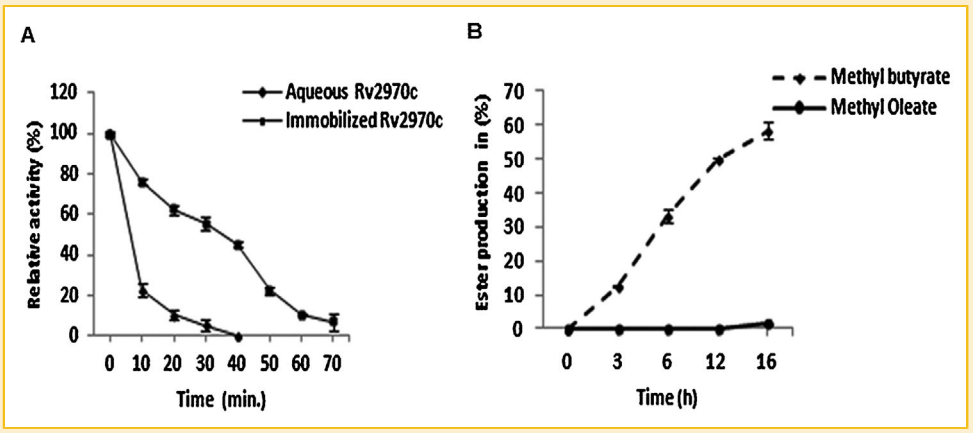

Fig. 5. Esterification studies with LipN. A: Comparison of thermostability between aqueous (32 U/ml) and 20 mg of immobilized LipN (2U/ mg of beads) enzyme at 60°C. B: Comparison of ester production between butyric acid and oleic acid with methanol directed by 200 mg of immobilized LipN (420 U) at different time intervals. (Each experiment was conducted independently three times in triplicate. Error bars indicate the standard deviation).

The enzyme did not show any positional specificity as it converted tributyrin into both mono and dibutyrin that is correlated with observation of inhibition of Lip N enzyme activity by both THL (a triacylglycerol lipase inhibitor) and RHC-80267 (a Diacylglycerol lipase inhibitor). Progress reaction curve (time dependent kinetic) study implies that LipN follow nonlipolytic carboxyl esterase like kinetic behavior while hydrolyzing small subtrates like pNP-butyrate and it doesn′t show latency phase demonstrated by lid containing lipases.

The conserved Ser216, Asp316 and His346 were confirmed as catalytic residues by site directed mutagenesis. Treatment of enzyme with NBS, a Trp modifier, resulted in complete loss of enzymatic activity. Structure analysis revealed that out of six Trp residues present in protein, Trp145 is less than 10 A° away from enzymatic catalytic cleft geometrically and is located in loop region, and could effectively influence substrate binding. In addition, it may serve as a contributor to both H-bond and aromatic–aromatic interaction in maintaining the cross-link within the interweaving framework of protein (Fig. S2). On substituting Trp145 to Ala, the mutant protein lost enzymatic activity completely which confirmed the involvement of Trp145, a non active site residue, to retain enzymatic activity. Similar role of non active site tryptophan present near catalytic triad helping in substrate binding to retain thioesterase-I enzyme activity was reported in *E. coli* [Lee et al., 2009]. This is the first report demonstrating the requirement of Trp residue for catalytic activity in enzyme belonging to HSL family in mycobacterium.

The cytosolic localization of LipN intrigued us to explore if LipN is able to carry out the esterfication reaction. The production of methyl butyrate esters of butyric acid and methanol by the immobilized LipN enzyme suggested the possible involvement of LipN in esterification processes in cell using short carbon chain fatty acids.

Expression of *rv2970c* gene was not observed during in-vitro growth conditions, but the gene was expressed in *M. tuberculosis* H37Ra infected macrophages. A multitude of stresses (i.e. oxidative, acidic, nutritive, nitrosative, hypoxic) have been reported to regulate the expression of several mycobacterial proteins with their subsequent involvement in the induction of nonreplicating persistence of *M. tuberculosis* during phagosome maturation arrest [Manganelliet al., 2004]. The up regulation of *rv2970c* gene expression under acidic condition intrigued us to seek a parallelism of *rv2970c* expression from the *lipF,* a well studied acid inducible Lip family member, which is not expressed under aerobic growth condition but showed acid induced response [Saviola et al., 2003; Richter et al., 2007]. We could locate in the upstream region of *rv2970c,* the -10 motif for SigA binding [sigma factor of LipF (TGTGAT)], with 2 base pair difference (TaTGcT) and a 68 bp putative promoter region [Rangannan and Bansal, 2011]. Regulatory networks (Chip-seq TbDtb) showed the presence of putative binding site of transcriptional regulator, Rv3736 of AraC/XylS-family, in the *rv2970c* gene. This family of genes is associated with multidrug resistance, acidic stress and organic solvent tolerance. Further, BLASTP analysis revealed that Rv3736 has significant homology with YdeO, GadW regulators of AraC/XylS family of *E. coli*. YdeO activated genes enable the *E. coli* to colonize animal intestines by contributing to adaptation to acidic conditions in the stomach [Yamanaka et al., 2014]. Moreover, the hexa-nucleotide repeat 5′-ATTTCA-3′(the YdeO box), a YdeO binding site, is conserved in LipN gene sequence in the vicinity of binding site of Rv3736. YdeO-regulated targets encode a hydrogenase and a quinone oxidase and stimulates acidic stress response. Therefore it puts forward a possibility that the expression of *rv2970c* might be induced under acidic conditions by YdeO regulon as acidic stress and quinone pool were reported to have direct correlations [Yamanaka et al., 2014]. GadW is also involved in regulation of the genes for glutamate-dependent acid resistance system. It′s expression is induced by YdeO [Yamanaka et al., 2014]. So the presence of binding sites of these acid stress responsive regulators could be instrumental in acid stress induction of LipN as well. Further detail studies are needed to confirm these bioinformatic analysis.

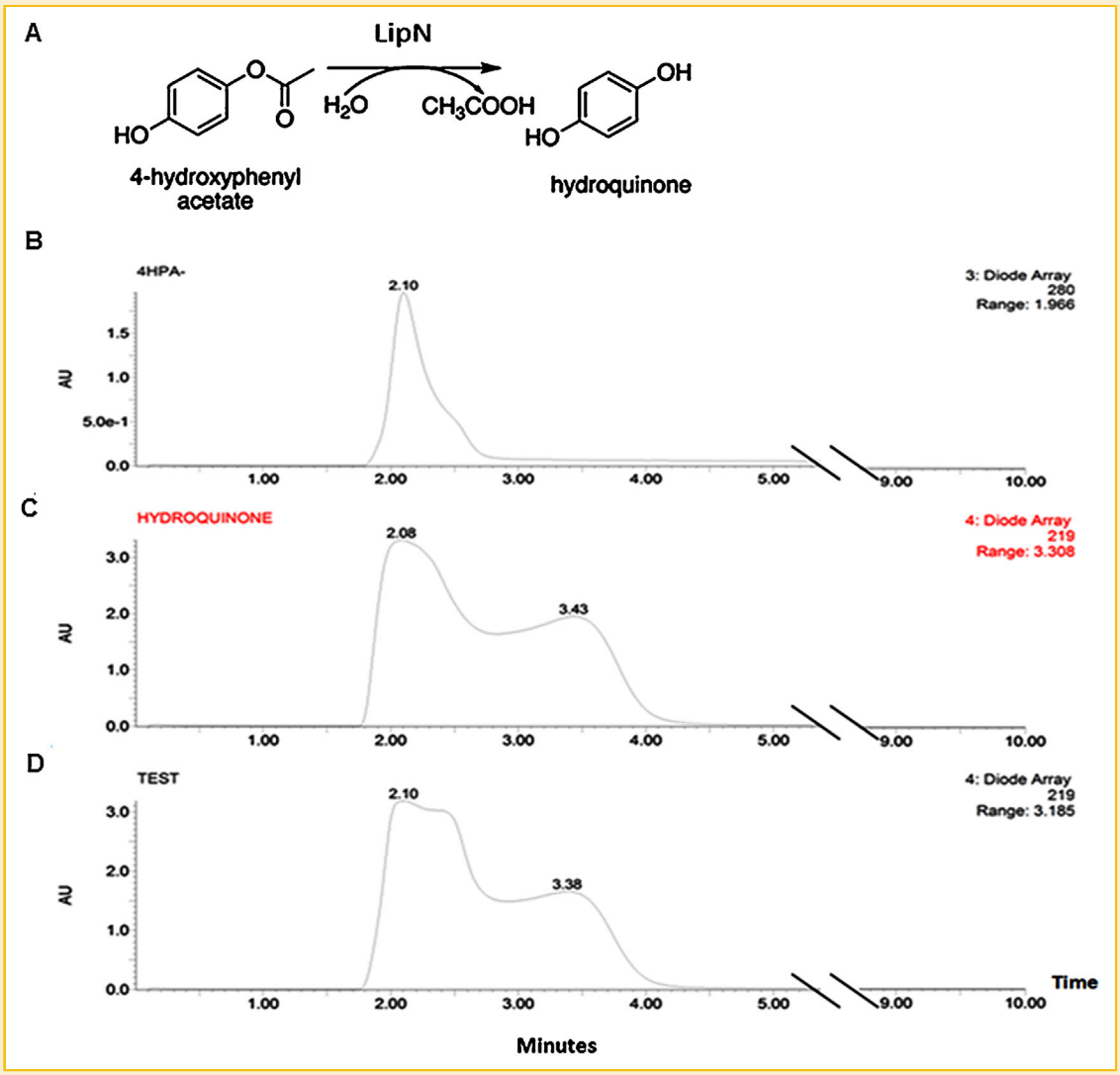

Fig. 6. LC analysis of 4-hydroxyphenylacetate hydrolase activity of LipN. A: Chemical reaction showing 4-hydroxyphenylacetate hydrolase activity of LipN. LC analysis of 4-hydroxyphenylacetate hydrolase activity of LipN at 280 nm and 219 nm. B: Analytical standard peak of 4-HPA with retention time 2.10 min, (C) Analytical standard LC analysis shows peak of hydroquinone showing retention time of 2.08 min and elbow formation of parabenzoquinone with retention time 3.43. D: 4-HPA reaction mix LC analysis after 2 h treatment with immobilized LipN (40U/20mg beads). Peaks for 4-HPA at 2.10 and product formed hydroquinone showing retention time of 2.08 min and elbow formation of parabenzoquinone with retention time 3.43 were observed. (Experiment was conducted independently three times).

In *E. coli*, it is reported that presence of phenolic acids confers acid stress. Activated universal stress response protein -1 (USP-1) dependent inactivation of padR, regulates padA to degrade phenolic acids in response to acid stress in *E. coli* [Gury et al., 2009]. Also in *E. coli*, 4-HPA degradation in particular is regulated by HpaA (p-hydroxyphenylacetic acid) regulator belonging to Arac/Xyls family which is induced under stress response in order to control carbon metabolism [Gallegos et al., 1997]. Similar kind of regulation might be operational in case of acid stress dependent induction of LipN (a 4-HPA hydrolase). However, further study is required to investigate the regulation mechanism behind acid stress induced expression of LipN.

A recent study showed LipN as one of the most prominent and consistently overexpressed protein in the proteomic analysis of rifampicin (RIF) and isoniazid (INH) drug resistant *Mycobacterium tuberculosis* clinical isolates from MDR patient and has suggested the possible use of LipN as a drug resistance marker [Singh et al., 2015] which is in corroboration to our observation of acid inducible expression of LipN and it's role in xenobiotic degradation.

In conclusion we demonstrated that LipN is an acid inducible protein localized in cytosolic fraction. It converted 4-hydroxyphenylacetate to hydroquinone. It is a non specific carboxyl ester hydrolase belonging to family IV carboxylesterases. In addition to Ser, Asp and His catalytic residues, it requires a Trp for enzyme activity. The 4-hydroxyphenylacetate hydrolase activity of Rv2970c might be instrumental in imparting resistance to the phagosomal acidic stress by regulating the quinone pool.

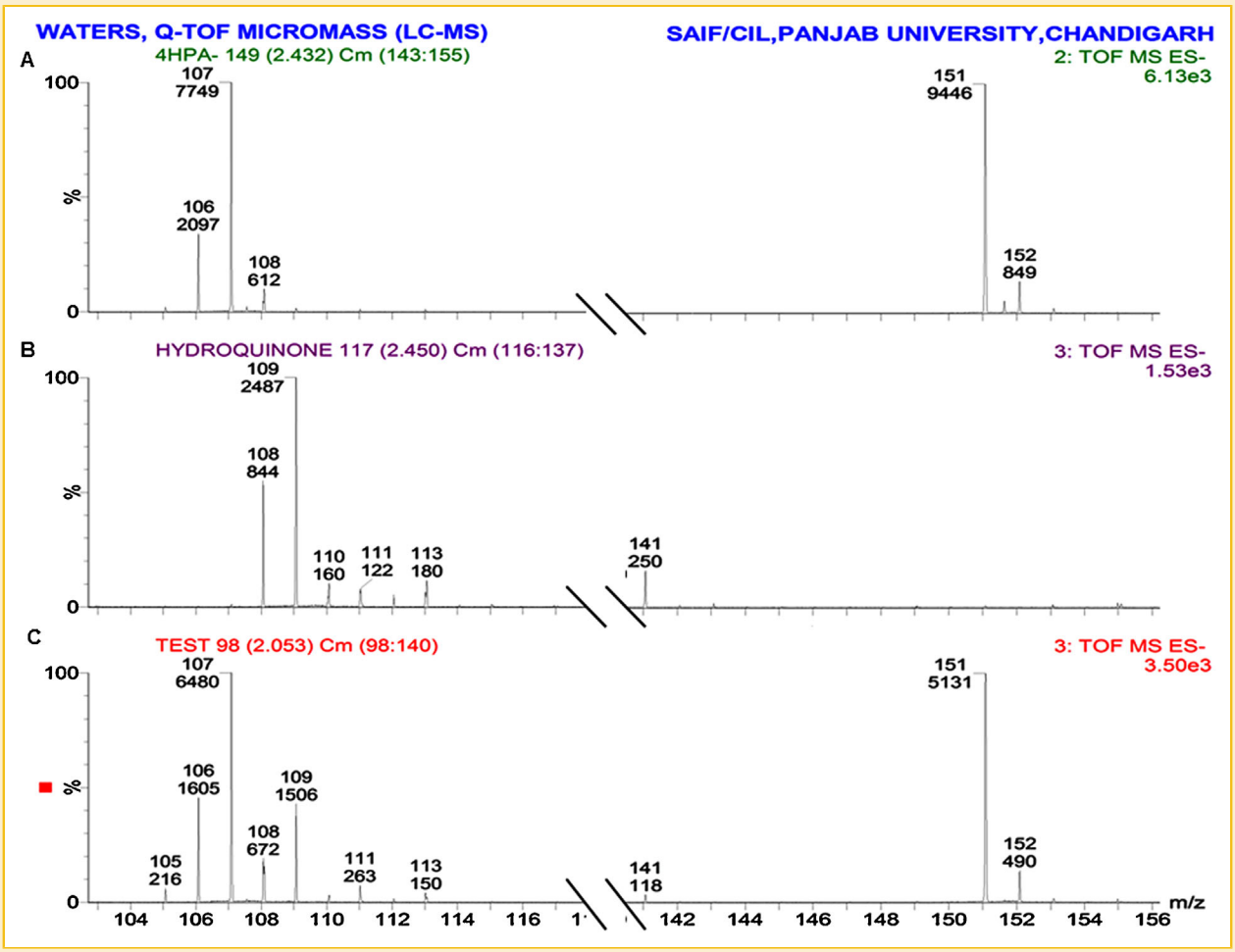

Fig. 7. TOF MS (ES–) analysis of 4–hydroxyphenylacetate hydrolase activity of LipN. A: Fragmentation pattern of Analytical standard 4–HPA with base peak at 151 m/z with ion intensity 9446 at 30V collision energy. B: Fragmentation pattern of analytical standard hydroquinone base peak was detected at 109 m/z with 2487 ion intensity at 30 V collision energy. C: 4–HPA reaction mix fragmentation pattern after 2 h treatment with immobilized LipN at 30 V collision energy.

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