

Cloning, Expression and Characterization of a Lipase Encoding Gene from Human Oral Metagenome

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Abstract The human oral metagenomic DNA cloned into plasmid pUC19 was used to construct a DNA library in *Escherichia coli*. Functional screening of 40,000 metagenomic clones led to identification of a clone LIP2 that exhibited halo on tributyrin agar plate. Sequence analysis of LIP2 insert DNA revealed a 939 bp ORF (*omlip1*) which showed homology to lipase 1 of *Acinetobacter junii* SH205. The *omlip1* ORF was cloned and expressed in *E. coli* BL21 (DE3) using pET expression system. The recombinant enzyme was purified to homogeneity and the biochemical properties were studied. The purified OMLip1 hydrolyzed *p*-nitrophenyl esters and triacylglycerol esters of medium and long chain fatty acids, indicating the enzyme is a true lipase. The purified protein exhibited a pH and temperature optima of 7 and 37 °C respectively. The lipase was found to be stable at pH range of 6–7 and at temperatures lower than 40 °C. Importantly, the enzyme activity was unaltered, by the presence or absence of many divalent cations. The metal ion insensitivity of OM-Lip1 offers its potential use in industrial processes.

Keywords Lipase · Human oral cavity · Metagenome · Biosensor

Introduction

The human body encompasses complex and dynamic microbial consortia. The human oral cavity in particular has more than 700 bacterial phylotypes, of which over 50 % have not been cultivated [1]. The unculturable population holds an enormous biotechnological potential but is difficult to study by classical microbiological methods. Recently, metagenomics—a culture independent approach has been employed to harness the gene pool of unculturable microorganisms. The oral cavity harbors a large and diverse microflora in the human body [2]. Hence, screening of the human oral metagenome should provide a rich source of novel biocatalysts.

Lipases are one of the most industrially important biocatalysts. The market demand for prokaryotic lipases is increasing at a high pace. Every industry expects lipase best suited for their process requirements. For example: detergent industry need alkaline lipase [3], bioremediation or bioaugmentation at low temperatures depend on cold active lipase [4], synthesis of biopolymers and biodiesels require thermotolerant lipase [5] and food, flavor industries require acidic lipase [6].

Lipase belongs to the α/β -hydrolase fold family of enzymes [7]. Lipase (E.C.3.1.1.3) has catalytic activity similar to esterase (E.C.3.1.1.1), but differs remarkably in its ability to hydrolyze glycerolesters with acyl chain lengths longer than 10 carbon atoms [5]. Bacterial lipolytic enzymes have been classified into eight different families based on their conserved amino acid sequences and biological properties [8]. In recent years, metagenomics has been successfully utilized to discover novel lipases from diverse environments such as Korean tidal flat sediment [9], cow rumen [10], peat-swamp forest soil [11] and waste water treatment plant [12]. The metagenomic studies of

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human oral cavity have been focused on understanding variation in microbial diversity with respect to host health status [13] and investigation of antibiotic resistance genes by sequencing technologies [14].

This is the first report on identification of a lipase from human oral metagenome by functional metagenomics approach. Further, biochemical characterization of this enzyme substantiates its possible use for industrial applications.

Materials and Methods

Strains, Plasmids and Chemicals

The plasmid vector pUC19 (Stratagene, USA) and the host *Escherichia coli* strain DH10B [Invitrogen (CA, USA)] were used for metagenomic library construction. For protein over expression, vector pET-30b and *E. coli* BL21 (DE3) from Novagen (CA, USA) were used.

Reagents for PCR, oligonucleotide primers, antibiotics, lipase substrates and all other biochemicals were from Sigma-Aldrich (St.Louis, MO, USA). T4 DNA ligase and restriction enzymes were obtained from MBI Fermentas (Opelstrasse, Germany).

Sampling

The study was conducted with approval of ethical committee of Madurai Kamaraj University. Participants were informed about the nature of experiment. A total of 110 volunteers representing both gender, in age group of 21–29 and not under antibiotic therapy for preceding 3 months were selected for the study. Participants were instructed not to brush their teeth, the night prior to sampling. Oral rinse with sterile water was collected in morning, before brushing. Immediately after sample collection, all the samples were pooled and processed for DNA isolation.

Metagenomic Library Construction and Functional Screening

Standard method for plasmid isolation, restriction enzyme digestion, competent cell preparation and transformation as described by Sambrook et al. [15] were followed.

Human oral metagenomic DNA was isolated using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) as per manufacture's protocol. The metagenomic DNA was partially digested with restriction enzyme *HindIII* and purified using QIAquick gel extraction kit (Qiagen, Hilden, Germany). The digested metagenomic DNA was ligated to *HindIII* digested and dephosphorylated pUC19 vector in a ratio 3:1 (insert:vector). The ligation mix was electroporated

into electrocompetent *E. coli* strain DH10B (200 Ω , 25 μ F and 2.5 kV) using Gene Pulser (Bio-Rad, USA). Transformants were grown at 37 °C overnight on Luria–Bertani (LB) agar containing 100 μ g/ml ampicillin, 20 μ g/ml 5-bromo-4-chloro-indolyl- β -D-galactopyranoside (X-gal), and 40 μ g/ml isopropyl- β -D-thiogalactopyranoside (IPTG).

Based on blue white screening the positive recombinant clones were selected. These metagenomic clones were cultured on LB agar supplemented with IPTG and 1 % tributyrin at 37 °C for 48 h. Thereafter, lipolytic activity was detected by zone of clearance around the positive clone [16].

Cloning, Overexpression and Purification of the Metagenomic Lipase

The open reading frame (ORF) encoding OMLip1, without stop codon was amplified from the recombinant plasmid of lipase positive metagenomic clone using forward primer Lip2F 5' - GCAAGCTTATGACAACAACGATGACTC-3' (*HindIII* site is underlined) and reverse primer Lip2R 5'- ATCTCGAGAGGAGTAGGTGCAGTCAC-3' (*XhoI* site is underlined). The PCR product was cloned into pET-30b vector and the resultant plasmid pETOMLip1 was then used to transform *E. coli* BL21.

LB medium with kanamycin (30 μ g/ml) was inoculated with 1 % (v/v) of overnight culture of *E. coli* BL21 carrying pETOMLip1 and grown aerobically at 37 °C until the culture reached an optical density of 0.4 at 600 nm. The culture was then induced by addition of 150 μ M of IPTG and grown with agitation at 200 rpm for 6 h at 37 °C. The induced cells were harvested by centrifugation at 4 °C for 10 min at 8,000 $\times g$ and washed with 50 mM Tris-buffer (pH 7.0). The cell pellet was resuspended in 10 volumes of buffer (50 mM Tris [pH 6.8], 0.5 M NaCl, 100 mM imidazole) and disrupted by sonication (four times for 30 s with 30 s interval) (Labsonic U, Germany).

The clarified cell lysate was applied onto a Bio-Scale Mini Profinity IMAC Cartridge (Bio-Rad, CA, USA) and eluted with imidazole gradient (100 mM–500 mM) following the manufacturer's recommendation. Fractions with lipolytic activity were pooled and dialyzed in 50 mM Tris buffer (pH 6.8) for 12 h.

Polyacrylamide Gel Electrophoresis (PAGE) and Zymogram Analysis

The proteins were resolved on native PAGE and SDS-PAGE as described by Laemmli [17]. The gel was stained with Coomassie brilliant blue R-250. The molecular mass of protein was determined by comparison with the standard protein marker (Fermentas, Opelstrasse, Germany) separated on the same gel. For zymogram analysis, the protein was separated on the SDS-PAGE. After electrophoresis, the

gel was kept at gentle shaking for 20 min in 20 % (vol/vol) isopropanol and for 10 min in distilled water at room temperature [18]. Subsequently, the gel was sandwiched on a petridish between the layers of LB agar containing 1 % tributyrin. The zone of clearance was clearly visualized after incubation for 2–3 h at 37 °C.

Enzyme Assay

Lipase activity was measured at 405 nm using assay procedure described by Winkler et al. [19]. The standard assay conditions used in this study measured lipase activity with 0.8 mM *p*-nitrophenyllaurate substrate in 50 mM Tris buffer (pH 6.8) after 10 min incubation at 37 °C. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of *p*-nitrophenol/min. All the assays were performed in triplicates.

Biochemical Characterization

Substrate specificity of the enzyme was analyzed under standard conditions using different fatty acid esters of *p*-nitrophenyl (*p*NP). Short acyl chain length substrate: *p*NP acetate (C2), medium acyl chain length substrate *p*NP caprylate (C8) and long acyl chain length substrates *p*NP laurate (C12), *p*NP myristate (C14) and *p*NP palmitate (C16) were examined.

Also hydrolytic activity of the purified enzyme towards triacylglyceride substrates triacetin (C2), tributyrin (C4), tricaprylin (C8), trimyristin (C14), tripalmitin (C16) and triolein (C18:1) was determined using titrimetric method described by Pinsirodom and Parkin [20] with slight modifications. Briefly, the reaction mix containing 10 mM substrate, 50 mM Tris buffer pH 7 and 1 % gum arabic was thoroughly emulsified. To this 20 ml of purified enzyme was added, mixed and the reaction was incubated at 37 °C for 30 min. The fatty acids released from triacylglyceride substrate during enzymatic hydrolysis were titrated to neutralization with 0.05 N NaOH in presence of thymolphthalein as an indicator. One unit of enzyme activity was defined as the amount of enzyme that released 1 µmol of fatty acid per min.

The pH optima was determined by measuring lipase activity using the standard assay conditions but varying the buffer pH from 3.0 to 11.0. To analyze pH stability, the residual enzyme activity was measured after incubating the enzyme with buffers of various pH in 1:4 ratio at room temperature for 24 h. The buffers used for pH dependence studies were 50 mM sodium acetate (pH 3.0–5.0), 50 mM Tris/HCl (pH 6.0–8.0), 50 mM sodium carbonate buffer (pH 9) and 50 mM glycine-NaOH (pH 10.0–11.0).

To evaluate the optimum temperature, lipase activity was measured under standard conditions at temperature range of 10–70 °C. The purified enzyme was incubated at

designated temperatures (10, 20, 30, 37, 40, 50, 60 and 70 °C) for 60 min and enzyme stability was measured using the assay described above.

For determining the effect of various modulators, the purified enzyme was pre-incubated with 0.5 % of detergent SDS (sodium dodecyl sulphate) and 5 mM of different metal ions (Mg^{2+} , Ca^{2+} , Mn^{2+} , Co^{2+} , Cu^{2+} , Zn^{2+} , Rb^{2+} , Hg^{2+} , Fe^{2+}), different inhibitors [ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), pepstatin A] and various reducing agents [dithiothreitol (DTT), β -mercaptoethanol (β -ME)] for 1 h at 37 °C. Thereafter, the residual activity in each case was measured under standard assay condition.

Bioinformatic Analyses

The open reading frames (ORFs) were identified by NCBI ORF finder program (<http://www.ncbi.nlm.nih.gov/projects/gorf/>) and were annotated using BLASTP similarity search against the NCBI non-redundant protein sequence database [21]. The multiple sequence alignment was performed with BioEdit version 7.0.5.3 software. The alternative structures of OMLip1 were generated using SWISS-MODEL [22]. These models were scored for various parameters by NIH SAVES server (<http://nihserver.mbi.ucla.edu/SAVES/>) and the best model was selected. The three dimensional structure was visualized and quality image was generated with PyMOL software (<http://www.pymol.org>).

Circular Dichroism (CD) Spectroscopy

CD spectroscopy analysis of the purified protein (0.085 mg/ml) in 10 mM sodium phosphate buffer (pH 7.0) was performed at room temperature using Jasco J-810 spectro-polarimeter (Tokyo, Japan). The CD spectrum was measured over a wavelength range of 190–240 nm, using a 0.1 cm path cell and a bandwidth of 1 nm. The collected spectrum was mean of two consecutive scans. The secondary structure of the protein was estimated from far-UV CD spectrum using the K2D3 algorithm [23].

Nucleotide Sequence Submission

The nucleotide sequence of LIP2 insert was deposited in the GenBank under the accession number JX154673.

Results and Discussion

A metagenomic library was constructed in *E. coli* DH5 α using human oral metagenomic DNA cloned into pUC19 vector. The library consisted of 4×10^4 clones with an average insert size of 5 kb. Screening of all these clones showed 20

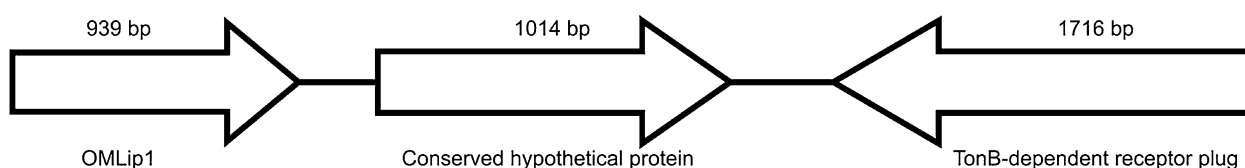


Fig. 1 Schematic diagram of gene organization in LIP2 insert DNA. The length of the genes and the protein product coded by each of them are shown

LIP2	-----	-----	-----	MTT	TMTQAAEIE	SFQNVLQQR	AWAGLQSKTL	KVGDITWSYS
A. junii SH205	-MMNRSFSTP	IRKILTAIAF	SCGLAATMTT	TMTQAAEIE	SFQNVLQQR	AWAGLQSKTL	KVGDITWSYS	
A. sp. NBRC 100985	MMNTLLPIP	FRKFTAFLL	SCSLG-AMAI	QTTQAAEIE	SFQNVLQQR	AWAGLQSKTL	KVGDITWSYS	
A. sp. V28	MMNTLLPIP	FRKFTAFLL	SCSLG-AMAI	QTTQAAEIE	SFQNVLQQR	AWAGLQSKTL	KVGDITWSYS	
A. parvus DSM 16617	-MMNHLFSTS	FRKILTAALI	SCSLA-TIAI	SSTQAAEIE	SFQNVLQQR	AWAGLQSKTL	KVGDITWSYS	
A. sp. NCTC 7422	MNTKNQFAMP	FRRILTAALL	ACSLT-TIAI	STTQAAEIE	SFQNVLQQR	AWAGLQSKTL	KVGDITWSYS	
A. sp. ATCC 27244	--MNIPFSLR	IAKFTATLL	ALSLT--ATI	NTTHAAEIE	SFQNVLQQR	AWAGLQSKTL	KVGDITWSYS	
LIP2	EGGQVGKPIV	VLIHGLAGSR	DNWNRVANAL	TGNYHVIIIPD	LPASGETQVP	KEFDYSVPNV	TEKLRRFIEA	
A. junii SH205	EGGQVGKPIV	VLIHGLAGSR	DNWNRVANAL	TGNYHVIIIPD	LPASGETQVP	KEFDYSVPNV	TEKLRRFIEA	
A. sp. NBRC 100985	EGGQAGKPIV	ILIHGLAGSR	DNWNRVAHAL	TANYHVIIIPD	LPASGETKVP	KEFDYSVPNV	TEKLRRFIEA	
A. sp. V28	DGGQAGKPIV	ILIHGLAGSR	DNWNRVAHAL	TANYHVIIIPD	LPASGETKVP	KEFDYSVPNV	TEKLRRFIEA	
A. parvus DSM 16617	EGGQAGKPIV	ILIHGLAGSR	DNWNRVARAL	TANYHVIIIPD	LPASGETQVP	KDFDYSIPNV	TEKLRRFIEA	
A. sp. NCTC 7422	EGGQVGKPIV	ILIHGLAGSR	DNWNRVARAL	TANYHVIIIPD	LPASGETQVP	KDFDYSVPNV	TEKLRRFIEA	
A. sp. ATCC 27244	EGGQSGKPII	VLIHGLAGSR	DNWNRVAHAL	TANYHVIIIPD	LPASGETQVP	KDFDYSVPNV	TEKLRRFIEA	
LIP2	ANLTGPAHIA	GHSLGGSIAM	LYAGQYPFET	KSLFLVDAAG	VYKAGNTPYL	KDPNQIRSMI	VSKKGDFNFI	
A. junii SH205	ANLTGPAHIA	GHSLGGSIAM	LYAGQYPFET	KSLFLVDAAG	VYKAGNTPYL	KDPNQIRSMI	VSKKGDFNFI	
A. sp. NBRC 100985	ANLTGPAHIA	GHSLGGSIAM	LYAGQYPFET	KSLFLIDAAG	VYKSATTPYL	KDPAQVKNMI	VSKKGDFNFI	
A. sp. V28	ANLTGPAHIA	GHSLGGSIAM	LYAGQYPFET	KSLFLIDAAG	VYKSATTPYL	KDPAQVKNMI	VSKKGDFNFI	
A. parvus DSM 16617	ANLTGPAHIA	GHSLGGSIAM	LYAGQYPFET	KSLFLIDAAG	VYKAATTPYL	KDPNHIKNMI	VSKKGDFNFI	
A. sp. NCTC 7422	ANLTGPAHIA	GHSLGGSIAM	LYAGQYPFET	KSLFLIDAAG	VYKSANTPYL	KDPTQVKNMI	VSKKGDFNFI	
A. sp. ATCC 27244	ANLTGPAHIA	GHSLGGSVAM	LYAGQYPFET	KSLFLVNAAG	VYRSANTPYL	KDPNQLRMNV	VSKKGDFNFI	
LIP2	MQQAMHNPPF	IPKEIAQAQE	KMMIGQIEQT	QKMVDQIIAL	NKLFTPDSFA	LLAKSIDAPT	LILWGKQDKI	*
A. junii SH205	MQQAMHNPPF	IPKEIAQAQE	KMMIGQIEQT	QKMVDQIIAL	NKLFTPDSFA	LLAKSIDAPT	LILWGKQDKI	
A. sp. NBRC 100985	MQQAMYSPPF	IPKEIAQAQE	KMMIGQVEET	QKMVDQIIAL	NKLFTPDSFA	LLAKSIDAPT	LILWGKQDKI	
A. sp. V28	MQQAMYSPPF	IPKEIAQAQE	KMMIGQVEET	QKMVDQIIAL	NKLFTPDSFA	LLAKSIDAPT	LILWGKQDKI	
A. parvus DSM 16617	MQQVMFTPPF	IPKEIAQAQE	KMMIGQAEQT	QKMVDQILVL	NKLYTPDSFA	LLARSIDAPT	LILWGKQDKI	
A. sp. NCTC 7422	MQQAMFTPPF	IPKEIAQAQE	KMMIGQVEQT	KKMVDQIIAL	NKLYTPDSFA	LLARSIDAPT	LILWGKQDKI	
A. sp. ATCC 27244	MQQAMYAPPF	IPKEIAQAQE	KMMIGQVEQT	KRMVDQVIAL	NKLYTPDSFA	LLARAIDAPT	LILWGKQDKI	
LIP2	INVEVAPELK	SLLKNAQAPV	ILDNVGHMPI	LEADQLVVQQ	YLPFLSKTQA	QKSVTAPT		
A. junii SH205	INVEVAPELK	SLLKNAQAPV	IMDNVGHMPI	LEADQLVVQQ	YLPFLSKTQA	QKSVTAPT		
A. sp. NBRC 100985	INVEVAPELK	SLLKNAQTPV	ILDNVGHMPI	LEADQLVVQQ	YLPFLSKIQA	QKPATAPSP		
A. sp. V28	INVEVAPELK	SLLKNAQTPV	ILDNVGHMPI	LEADQLVVQQ	YLPFLSKIQA	QKPATAPSP		
A. parvus DSM 16617	INVEVAPELK	SLLKNAQTPV	ILDNVGHMPI	LEADQLVVQQ	YLPFLNKIQS	QKPVTAAPT		
A. sp. NCTC 7422	INVEVAPELK	SLLKNAQTPV	ILDNVGHMPI	LEADQLVVQQ	YLPFLSKIQV	QKPATSSSP		
A. sp. ATCC 27244	INVEVAPELK	SLLKNAQAPV	ILENVGHMPI	LEADQLVVQQ	YLPFLSKIQA	QKPATASTP		

Fig. 2 Multiple sequence alignment of lipase from metagenomic clone LIP2 and its homologs. The accession number of amino acid sequences used are as follows: ZP_06065291, lipase from *Acinetobacter junii* SH205; ZP_09220445.1, putative hydrolase from *Acinetobacter* sp. NBRC 100985; AEP27110.1, esterase from *Acinetobacter* sp. V28; ZP_11043973.1, lipase from *Acinetobacter*

parvus DSM 16617; ZP_10860703, lipase from *Acinetobacter* sp. NCTC 7422; ZP_03822931.1, alpha/beta superfamily hydrolase/acyltransferase from *Acinetobacter* sp. ATCC 27244. Identical residues highlighted in gray background. The rectangle depicts the conserved pentapeptide motif. The star represents amino acid residues forming the catalytic triad

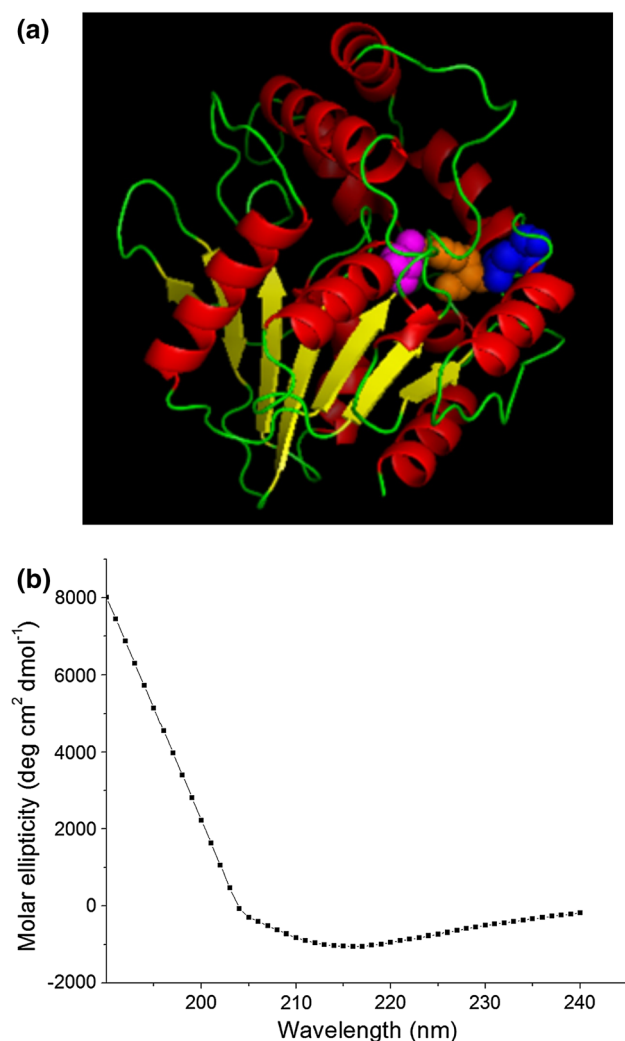


Fig. 3 Structure prediction and CD spectroscopic analysis of OMLip1. **a** The in silico structure of OMLip1 showing the catalytic residues Ser101 (magenta), His147 (orange) and Asp213 (blue). α -helices are represented as red coils, β -sheets as yellow arrows and loops in green. **b** The far-UV CD spectrum of OMLip1 showing the molar ellipticity measured from 190 to 240 nm. (Color figure online)

independent clones that exhibited a zone of hydrolysis on LB agar plate containing 1 % tributyrin. All the lipolytic clones harboured the same insert of ~ 3.9 kb, suggesting that this genomic segment is quite abundant in tested metagenomic DNA sample. The recombinant plasmid pUCLip2 from a selected lipolytic clone LIP2 was retransformed into new DH10B host and appearance of lipolytic transformants validated the presence of trait on plasmid. The hydrolytic activity of LIP2 could only be detected in presence of IPTG.

Sequence Analysis

Both strands of 3,944 bp insert DNA from pUCLip2 were completely sequenced. The NCBI ORF finder found that the

insert sequence harbored three ORFs (Fig. 1). A BLAST search in NCBI protein database showed that the 939 bp ORF named *omlip1* showed 99 % identity to lipase1 gene of *Acinetobacter junii* SH205, though there are no reports on functional characterization of this enzyme. Multiple sequence alignment of OMLip1 with other closely related lipase revealed the conserved pentapeptide motif GHSLG (Fig. 2). Pfam analysis of OMLip1 indicated the catalytic triad consisting of a nucleophile Ser126 located within the pentapeptide, Asp251 and His280. These results indicated that OMLip1 belongs to family I of bacterial lipases.

The 3D structure of OMLip1 was built based on homology modelling and the constructed model was validated by Ramachandran plot. The plot indicated that 88 % of the amino acid residues were scattered within the most favoured regions, 10.7 % residues were in the allowed regions and only 1.3 % residues were in the disallowed regions. *In silico* model of OMLip1 showed that the helical content of the enzyme was more than the β -sheet structure. The conserved strand-nucleophile-helix feature of α/β hydrolase fold family, termed the nucleophile elbow, has been observed. The main chain phi and psi angles of Ser126 are outside the favoured regions of Ramachandran plot which is another common feature observed in the nucleophile backbone of α/β hydrolase fold family. The distantly placed catalytic residues in the primary structure come closer in the predicted tertiary structure (Fig. 3a).

Figure 3b shows the CD spectra of OMLip1. The analysis of CD spectra data showed that the secondary structure of OMLip1 contained 25.95 % α -helices and 20.22 % β -strands. The results from CD studies are in accordance with the predicted three dimensional structure of OMLip1.

Heterologous Expression and Purification of OMLip1

The ORF coding for OMLip1 was cloned in pET-30b and transformed into *E. coli* BL21 (DE3). When BL21 harbouring the resultant plasmid pETOMLip1 was induced with IPTG, the functional enzyme was produced. Some members of bacterial lipase family I are dependent on chaperone protein called lipase-specific foldase (Lif) for expression of active enzyme [8]. However, OMLip1 belonging to this family showed Lif independent activity.

The hexahistidine-tagged OMLip1 was purified by Ni-NTA affinity chromatography. The purified OMLip1 appeared as a single protein band of same molecular weight on both native PAGE and SDS-PAGE gels (Fig. 4a, Fig. 4b). Also, at the corresponding position in the zymogram a zone of tributyrin hydrolysis was observed. The results confirmed that the purified OMLip1 was a monomeric protein with a molecular mass of 41 kDa.

Fig. 4 PAGE analysis. **a** Native PAGE. Lane M molecular weight marker, Lane 1 purified OMLip1. **b** SDS-PAGE. Lane M molecular weight marker, Lane 1 intracellular lysate of *E. coli* BL21 (DE3) transformed with pET-30b, Lane 2 intracellular lysate of *E. coli* BL21 (DE3) transformed with pETOMLip1, Lane 3 purified fraction from Ni-NTA chromatography, Lane 4 zymogram of purified fraction with tributyrin as substrate

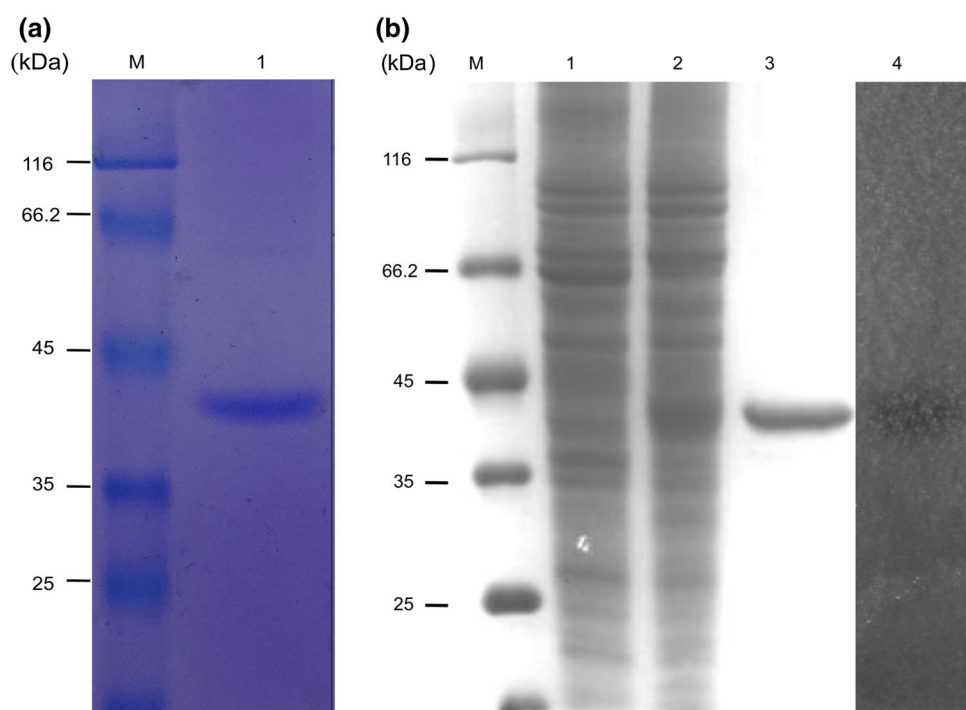
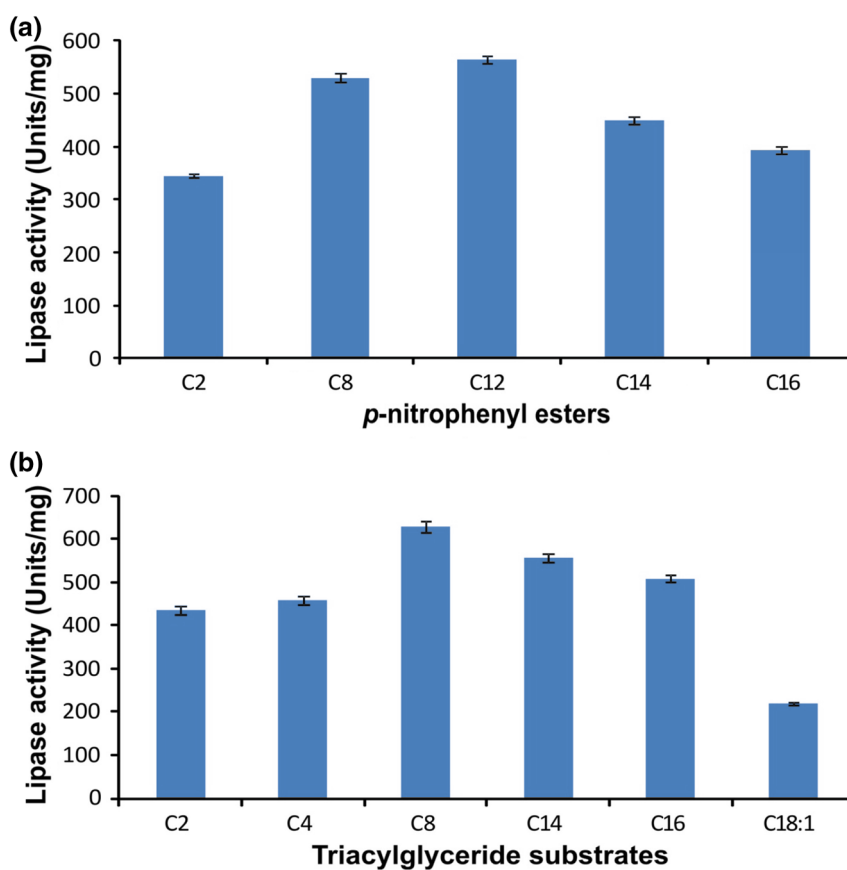


Fig. 5 Specific activity of OMLip1 towards **a** *p*-nitrophenyl esters and **b** triacylglycerol esters with different chain length fatty acids. Activities were measured by spectrophotometric assay for *p*-nitrophenylesters and with titrimetric method for triacylglycerides. Error bars show standard error between three observations



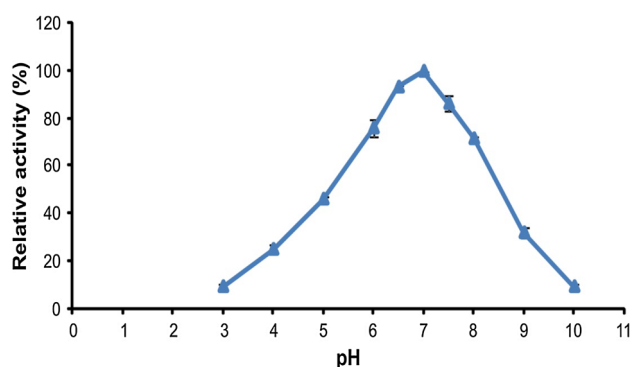


Fig. 6 Lipase activity plotted as a function of varying pH. Values were mean of triplicates and expressed in percentage (%) relative to maximal activity

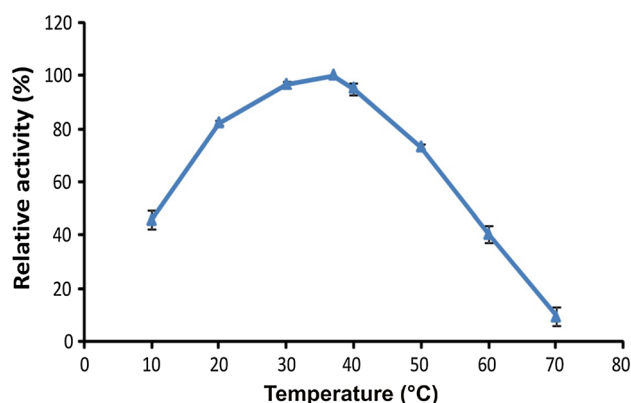


Fig. 7 Effect of temperature on the activity of OMLip1. The highest value was considered as 100 % and accordingly relative activity at mentioned temperatures were calculated

Substrate Specificity

The catalytic activity of OMLip1 towards various *p*NP esters was determined (Fig. 5a). The medium and long chain fatty acid esters were more preferred substrates than ester of short chain fatty acid. The enzyme showed maximum specific activity towards *p*NP laurate (C12). Furthermore the hydrolytic activity of OMLip1 towards various triglycerides was measured (Fig. 5b). The enzyme showed highest hydrolytic activity towards medium chain triglyceride (C8) followed by long chain triglycerides (C14, C16) and short chain triglycerides (C2, C4). Lipase resembles esterase in catalytic activity but only the former has the capability to hydrolyze water-insoluble fats containing medium to long chain fatty acyl chains [24]. The catalytic profile of OMLip1 with *p*NP substrates and triacylglycerol substrates confirms the enzyme as a true lipase.

Effect of Temperature and pH on Lipase Activity and Stability

The purified lipase exhibited highest stability at pH 6.5 after 24 h of incubation. At pH below 6.0 and above 7.0 the enzyme stability was drastically altered. Lipase activity was maximal at pH 7.0 (Fig. 6). The enzyme was stable up to 37 °C. The enzyme stability gradually decreased above 40 °C with a half life of 20 min at 60 °C. The purified protein exhibited maximum enzyme activity at 37 °C (Fig. 7). The pH optima and temperature optima of this enzyme emphasizes the fact that it is suited to perform excellently in human salivary pH range of 6.4–7.0 and average human body temperature of 37 °C.

Effect of Modulators

Addition of PMSF and pepstatin A reduced the lipase activity to 76 % and 65 % respectively. The decrease of lipase activity in the presence of PMSF and pepstatin A indicated that OMLip1 has active serine and aspartate residues in its catalytic site. Upon incubation of OMLip1 with anionic detergent SDS (1 %) the lipase activity was reduced by 50 %. Salameh et al. [25] showed that SDS promotes conformational changes in lipases from *Thermosyntropha lipolytica*. Unfavorable conformational changes induced by SDS in OMLip1 might be attributed for the observed drop in lipase activity. The enzyme activity was unaffected by addition of reducing agents DTT or β-ME. Sequence

Table 1 Effect of various modulators on lipase activity

Modulator	Relative activity (%)
None	100
PMSF (5 mM)	76
Pepstatin A (5 mM)	65
EDTA (5 mM)	101
DTT (5 mM)	99
β-ME (5 mM)	100
MgCl ₂ (5 mM)	92
CaCl ₂ (5 mM)	99
MnCl ₂ (5 mM)	101
CoCl ₂ (5 mM)	105
CuCl ₂ (5 mM)	96
ZnCl ₂ (5 mM)	101
RbCl ₂ (5 mM)	106
HgCl ₂ (5 mM)	85
SDS (1 %)	50

Purified OMLip1 was preincubated with the listed additives for 1 h at 37 °C and the residual activity was estimated by standard assay procedure

Lipase activity expressed as percentage (%) relative to the control (with no additive)

analysis of this enzyme shows it has no cysteine residues. As the protein lacks disulphide bridges, addition of reducing agents has no effect on its activity. Disulphide bridges are the strongest interaction determining the secondary structure of a protein. In human oral milieu the pH, temperature and ion flux is variable, functionality of an enzyme is ensured by not having such strict bonds but more flexible hydrogen bonds and vanderwall interactions [26].

The addition of Ca^{2+} had little effect on lipase activity (99 %). Remineralization and demineralization constantly changes calcium ion concentration in human oral cavity. OMLip1 is particularly well suited to the environment of human oral cavity as unlike most bacterial lipases it is not activated by calcium ions. Among the other tested divalent ions a marginal decrease in lipase activity was observed upon incubation with ions Mg^{2+} , Cu^{2+} whereas Rb^{2+} , Co^{2+} , Mn^{2+} , Zn^{2+} caused a slight increase in activity (Table 1). But Hg^{2+} showed a noticeable negative effect. Cations are known to have activating/inhibiting effect on lipase activity. But activity of OMLip1 was not affected by presence or absence of most of the divalent ions. Further this property of OMLip1 is substantiated by the observation that upon addition of divalent metal ion chelator EDTA no apparent change in lipase activity was observed.

Lipase based biosensors are employed for quantitative determination of lipids in clinical diagnosis, food industry and environmental monitoring [27]. The biosensor has a lipase moiety which acts on the lipids in the analytical sample and the released biological signal is converted by the coupled transducer into an optical/electrochemical signal. Accurate estimation of lipids in body fluids, food samples or environmental samples by enzymatic biosensor is modulated by presence of metal ions. Insensitivity of OMLip1 to several metal ions makes it an ideal candidate in development of lipase based biosensor for precise estimation of lipid content, irrespective of other factors.

In conclusion, the biochemical characteristics (lack of cysteine residues, tolerance to fluctuation of metal ions concentration and pH and temperature optima) of OMLip1 identified from human oral metagenome are vital factors contributing towards protein stability and activity in this niche. Along with the advantageous features, the propensity of high occurrence in metagenome raises an important question regarding the context and relevance of this enzyme in human oral perspective.

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References

1. Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE (2005) Defining the normal bacterial flora of the oral cavity. *J Clin Microbiol* 43:5721–5732
2. Wilson M (2005) *Microbial Inhabitants of Humans; Their Ecology and Role in Health and Disease*. Cambridge University Press, Cambridge
3. Sharma R, Chisti Y, Banerjee UC (2001) Production, purification, characterization, and applications of lipases. *Biotechnol Adv* 19:627–662
4. Suzuki T, Nakayama T, Kurihara T, Nishino T, Esaki N (2001) Cold-active lipolytic activity of psychrotrophic *Acinetobacter* sp. strain no. 6. *J Biosci Bioeng* 92:144–148
5. Rhee JK, Ahn DG, Kim YG, Oh JW (2005) New thermophilic and thermostable esterase with sequence similarity to the hormone-sensitive lipase family, cloned from a metagenomic library. *Appl Environ Microbiol* 71:817–825
6. Mhetras NC, Bastawde KB, Gokhale DV (2009) Purification and characterization of acidic lipase from *Aspergillus niger* NCIM 1207. *Bioresour Technol* 100:1486–1490
7. Holmquist M (2000) Alpha/Beta-hydrolase mechanisms fold enzymes: structures, functions and mechanisms. *Curr Protein Pept Sci* 1:209–235
8. Arpigny JL, Jaeger KE (1999) Bacterial lipolytic enzymes: classification and properties. *Biochem J* 343:177–183
9. Lee MH, Lee CH, Oh TK, Song JK, Yoon JH (2006) Isolation and characterization of a novel lipase from a metagenomic library of tidal flat sediments: evidence for a new family of bacterial lipases. *Appl Environ Microbiol* 72:7406–7409
10. Liu K, Wang J, Bu D, Zhao S, McSweeney C, Yu P, Li D (2009) Isolation and biochemical characterization of two lipases from a metagenomic library of China Holstein cow rumen. *Biochem Biophys Res Commun* 385:605–611
11. Bunterngsook B, Kanokratana P, Thongaram T, Tanapongpipat S, Uengwetwanit T, Rachdawong S, Vichitsoonthonkul T, Eurwilaichitr L (2010) Identification and characterization of lipolytic enzymes from a peat-swamp forest soil metagenome. *Biosci Biotechnol Biochem* 74:1848–1854
12. Glogauer A, Martini VP, Faoro H, Couto GH, Müller-Santos M, Monteiro RA, Mitchell DA, de Souza EM, Pedrosa FO, Krieger N (2011) Identification and characterization of a new true lipase isolated through metagenomic approach. *Microbiol Cell Fact* 10:54–67
13. Belda-Ferre P, Alcaraz LD, Cabrera-Rubio R, Romero H, Simón-Soro A, Pignatelli M, Mira A (2012) The oral metagenome in health and disease. *ISME J* 6:46–56
14. Warburton P, Roberts AP, Allan E, Seville L, Lancaster H, Mullany P (2009) Characterization of *tet*(32) genes from the oral metagenome. *Antimicrob Agents Chemother* 53:273–276
15. Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York
16. Lee SW, Won K, Lim HK, Kim JC, Choi GJ, Cho KY (2004) Screening for novel lipolytic enzymes from uncultured soil microorganisms. *Appl Microbiol Biotechnol* 65:720–726
17. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685
18. Sommer P, Bormann C, Götz F (1997) Genetic and biochemical characterization of a new extracellular lipase from *Streptomyces cinnamonomeus*. *Appl Environ Microbiol* 63:3553–3560
19. Winkler UK, Stuckmann M (1979) Glycogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens*. *J Bacteriol* 138:663–670
20. Pinsirodom P, Parkin KL (2001) Lipase assays. In: *Current protocols in food analytical chemistry*, John Wiley and Sons, Inc., pp C3.1.1–C3.1.13

21. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215:403–410
22. Arnold K, Bordoli L, Kopp J, Schwede T (2006) The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics* 22:195–201
23. Jeune CL, Navarro MAA, Iratxeta CP (2011) Prediction of protein secondary structure from circular dichroism using theoretically derived spectra. *Proteins* 80:374–381
24. Snellman EA, Sullivan ER, Colwell RR (2002) Purification and properties of the extracellular lipase, LipA, of *Acinetobacter* sp. RAG-1. *Eur J Biochem* 269:5771–5779
25. Salameh MA, Wiegel J (2010) Effects of detergents on activity, thermostability and aggregation of two alkalithermophilic lipases from *Thermosyntropha lipolytica*. *Open Biochem J* 4:22–28
26. Pollock MR, Richmond MH (1962) Low cyst(e)ine content of bacterial extracellular proteins: its possible physiological significance. *Nature* 194:446–449
27. Benjamin S, Pandey A (1998) *Candida rugosa* lipases: molecular biology and versatility in biotechnology. *Yeast* 14:1069–1087