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Isolation and Characterization of Novel Lipase Gene *LipHim1* from the DNA Isolated from Soil Samples

Pavan Kumar Pindi*, **Raja Srinath R., and Theetha L. Pavankumar**

Department of Microbiology, Palamuru University, Mahabubnagar 509001, Andhra Pradesh, India

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Metagenomics is a magnificent tool to isolate genes from unknown/uncharacterized species and also from organisms that cannot be cultured. In this study, we constructed a metagenomic library from isolated DNA of soil samples collected from Palamuru University campus premises, in Mahabubnagar district of Andhra Pradesh, India. We isolated a novel lipase gene *LipHim1*, which has an open reading frame of 591 base pairs and encodes ~23 kDa protein consisting of 196 amino acids. The Lipase LipHim1 showed maximum 32% homology at the protein level with the extracellular *Aeromonas hydrophila* lipase (Class II, GDSL family) and was significantly different from all other known lipases. The isolated lipase catalyzed the hydrolysis of fatty acid esters of polyoxyethylene sorbitan such as Tween 60. Our results indicate that the isolated lipase gene is novel.

Keywords: metagenomic library, lipase, *LipHim1* gene, Tween 60

Introduction

Lipolytic enzymes such as esterases (EC3.1.1.1) and lipases (EC3.1.1.3) catalyze both the fat hydrolysis and the synthesis of fatty acid esters including acylglycerides as biocatalysts (Jaeger *et al*., 1999). Bacterial diversity is remarkable at no less than 10^3–10^6 distinct prokaryote taxa per gram of pristine soil sediments (Torsvik *et al*., 1990) that possibly contributes to the presence of large number of genes that encode for several metabolic enzymes having great industrial, biotechnological and pharmaceutical importance. Screening of such genes, based on the function or sequence identity have led to the identification of numerous distinct enzymes from metagenome libraries of various environments such as soil, water, sediment, and extreme environments (Handelsman, 2004; Lorenz and Eck, 2005; Schmeisser *et al*., 2007). Lipases are highly exploited enzymes in the biotechnology industry and there are constant efforts to improve the catalytic activity and stability by directed evolution. Lipase is a versatile enzyme and one of the most important biocatalysts used in laundry soap, food, chemicals, and also in pharmaceutical industries (Jaeger and Eggert, 2002; Gupta *et al*., 2004). Another significant source of information about novel enzymes and their origin are extremophiles, which strive in harsh environmental conditions. Isolation of enzymes from extremophiles is incredibly valuable as they retain their catalytic activity even at the extreme temperatures. In this study, we isolated a lipase gene from a metagenomic library constructed from the DNA of soil samples. Here, we demonstrate its catalytic ability to hydrolyze the fatty acid esters of polyoxyethylene sorbitan such as Tween 60. The isolated lipase showed 32% identity with *A. hydrophila* and does not seem to associate with any other existing lipase family (Arpigny and Jaeger, 1999).

Materials and Methods

Sample collection and DNA extraction

DNA was extracted from the soil samples collected from Palamuru University campus premises in Andhra Pradesh, India, by the methods described earlier (Zhou *et al*., 1996). Briefly, finely sieved 3 g of soil was mixed with 6 ml of extraction buffer (100 mM Tris-Cl; pH 8.0, 100 mM Na-EDTA; pH 8.0, 1.5 M NaCl). After gentle mixing, 0.5 mg of proteinase K was added, and incubated at 37°C with gentle shaking at 180 rpm for 30 min. For efficient cell lysis, 3 ml of 20% SDS was added and incubated at 65°C for 90 min. Further, three cycles of freezing in liquid nitrogen and thawing at 65°C in water bath were conducted to release DNA from the microbial cells in the soils. The samples were centrifuged and supernatant was treated with 30% Polyethylene glycol and 1.6 M NaCl in 1:1 ratio for 12 h and centrifuged. Resulting supernatant was phenol-chloroform extracted and DNA was precipitated by ethanol. DNA pellet was air-dried and dissolved in TE buffer.

Construction of metagenomic library

Approximately 1 mg of isolated DNA was partially digested using *Bam*HI restriction enzyme. These partially digested *Bam*HI fragments were cloned into a copy control vector pCC1BAC (Epicentre, USA) as described by manufacturer's protocol. In brief, *Bam*HI DNA fragments ranging from 2 to 20 kb in size were ligated with the pCC1BAC vector (Table 1) and transformed in to EPI300 *E. coli* strain (Epicentre) and grown overnight on LB medium containing 25 mg/ml of chloramphenicol. The resulting library was stored in LB media containing 15% glycerol in 396-well storage plate at

*For correspondence. E-mail: pavankumarpindi@gmail.com; Tel.: +91-8542-275104; Fax: +91-8542-221020

Table 1. Plasmids used in this study

Plasmids	Description	References
pCC1BAC	Copy control BAC plasmid predigested with *Bam*HI	EPICENTRE
pBacLip	Derivative of pCCIBAC containing ~2.8 kb of metagenomic DNA fragment	This study
pBKS-Lip	pBluescript vector containing ~2.8 kb of DNA fragment having putative lipase gene	This study
pET28b	Protein expression vector with Kanamycin marker	Novagen
pET-Lip	Derivative of pET 28b containing 591 bp of lipase gene in-frame with His-tag at *Nde*I and *Sac*I sites.	This study

-80°C. Using this protocol, we generated a large insert Bacterial Artificial Chromosome (BAC) library of metagenomic DNA consisting of 3000 clones.

Screening of metagenomic library for lipase activity

The metagenomic library was screened for lipase genes by streaking metagenomic BAC clones on a Luria-Bertani (LB) agar plate containing Tween 60 (2%), chloramphenicol (25 mg/ml) and incubated at 37°C for 24 h. In the presence of Tween 60, lipases are capable of hydrolyzing fatty acid esters of polyoxyethylene sorbitan (Tweens) leaving turbidity on LB agar plate (Emanuilova *et al.*, 1993). Based on the turbidity or opacity formed on LB agar plate containing Tween 60, ~3000 clones of metagenomic library were screened for the lipase activity.

DNA sequencing and identification of lipase gene

pBluescript KS (pBKS) vector containing lipase gene (pBKS-Lip) was utilized for DNA sequencing. The complete 2.8 kb DNA region of interest using a series of primers (Bio-serve, India) such as M13F, LFP2, PPF, PPR, and M13R was sequenced as indicated in Table 2. The DNA sequences were assembled using the BIOEDIT program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The complete DNA sequence was assembled and analyzed using the tools BLASTN, BLASTX, and ORF-finder (NCBI) (http://www.ncbi.nlm.nih.gov). The identified translated gene was compared for percentage identity with known lipase genes. ClustalW2 program was used for protein sequence alignment (www.ebi.ac.uk).

Protein expression and purification of lipase enzyme

The 591 bp fragment of putative lipase gene was PCR amplified by using specific primer LFNd1 and LRS1, which contain *Nde*I and *Sac*I restriction sites, respectively (Table 1). The PCR amplified DNA fragment was digested using *Nde*I and *Sac*I restriction enzymes and cloned in-frame with 6X His-tag of pET28b vector (Novagen, Germany). The pET vector containing lipase gene (pET-Lip) was further transformed into suitable bacterial expression system for expression and purification of lipase.

Assay for lipase activity of extra cellular lipase expressed by pET-Lip

In order to assess the extracellular lipolyitc activity, the lipase was over expressed in BL21 (DE3) codon plus RIL (Stratagene, USA) cells harboring pET-Lip vector by IPTG induction. After 4 h of IPTG induction cells were centrifuged, both bacterial pellet and culture supernatant were collected. Later, the culture supernatant, presumably containing extracellular lipase was added into the holes made on LB agar plates containing Tween 60 and incubated at 37°C for 12 h. A zone of appearance of turbidity around spots inoculated with supernatant was measured as a function of lipolytic activity of extracellular lipase.

Results

Construction of metagenomic library and screening for clones

DNA was extracted from the soil samples as described in

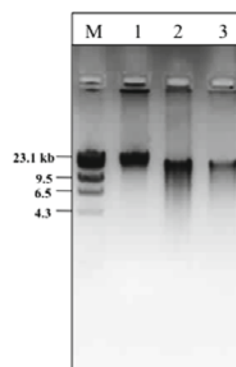

Fig. 1. Restriction digestion of isolated DNA with *Bam*HI. DNA isolated from soil samples was restriction digested with *Bam*HI enzyme. Lanes: 1, contains undigested DNA; 2–3, contain *Bam*HI digested DNA.

Table 2. Primers used in this study

Primers	Sequence (5′→3′ direction)	Related information
M13R	AGCGGATAACAATTTCACACAGGA	Reverse primer used for sequencing
M13F	CGCCAGGGTTTTCCCAGTCACGAC	Forward primer used for sequencing
LFP-2	CGACCTACTGCCCTGCCACTACTCG	Forward primer used for sequencing
PPF	GGTGCTGAATCAGGAGGAAC	Forward primer used for sequencing
PPR	GAGTGCATTCCTGAGCCTTC	Reverse primer used for sequencing
LFNd1	GCATGGCATATGTTCCTCCTGATTCAGCAC	Forward primer with *Nde*I site (underlined) used for amplification of *LipHim1* gene
LRS1	CAGATGAGCTCTCAGGCGGTCAGGCTCTCG	Reverse primer with *Sac*I site (underlined) used for amplification of *LipHim1* gene

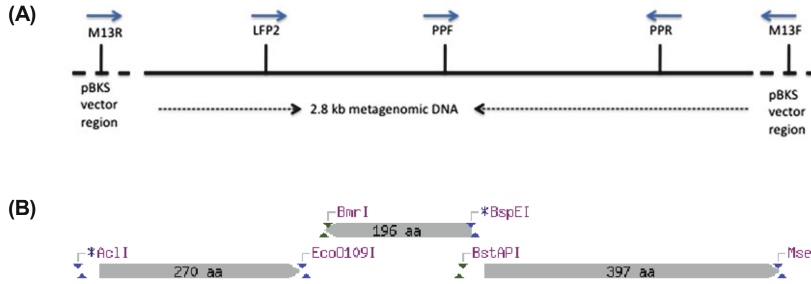

Fig. 2. Organization cloned 2.8 kb of metagenomic DNA. (A) schematic representation of location and direction of the primers used for sequencing 2.8 kb of metagenomic (B) Identification of open reading frames (ORF) within 2.8 kb DNA sequence.

materials and methods. DNA was further purified by Sephadex G-50 columns to get rid of impurities like humic acid. The isolated DNA quality and integrity was confirmed by agarose gel electrophoresis (0.8%) (Fig. 1, Lane 1). The DNA was restriction digested with *Bam*HI enzyme to get smaller DNA fragments ranging from 2 to 20 kb of size (Fig. 1, Lanes 2 and 3). These *Bam*HI digested fragments were then cloned into copy control vector pCC1BAC (Epicentre) and a metagenomic library was generated.

The metagenomic library containing 3000 BAC clones was screened for lipase activity. We found six metagenomic clones showing lipase activity on Tween 60 containing LB agar plates (data not shown). Among the six putative lipase expressing BAC clones, we selected one clone (BacLip-196) having robust lipase activity for further analysis. Plasmid DNA was isolated from the BacLip-196 clone and digested with *Bam*HI to release the DNA fragment containing putative lipase gene. The released DNA fragment size was found to be 2.8 kb in size. For the convenience of DNA sequencing, the 2.8 kb DNA fragment was sub cloned into the *Bam*HI site of

(B)

5'3' Frame 1

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atgttcctcctgattcagcaccgcactccggtcatcggagcatccgctgcgcttgtgccc
 M  F  L  L  I  Q  H  R  T  P  V  I  G  A  S  A  A  L  V  P
gtgctctcacccgcgccgctacgccgccgcgcgcgatctgtgcgctctgctgtccctg
 V  L  S  P  A  P  L  R  R  R  A  R  D  L  C  A  L  L  S  L
gccctgctctgcctggctggatgcaagcccgctggctccgacgatgccgcccccgccccg
 A  L  L  C  L  A  G  C  K  P  A  G  S  D  D  A  A  P  A  P
tcagcggctgggccgtccccggctgccgctcccacagcttcgagcgctccgggtgcttcg
 S  A  A  G  P  S  P  A  A  A  P  T  A  S  S  A  P  G  A  S
gaccaggccgcatcccccgccactccgctcgagacctgcctcacccaggtctctgtcgat
 D  Q  A  A  S  P  A  T  P  L  E  T  C  L  T  Q  V  S  V  D
ccgacctccgctgtgaccgccttcctggaactcgacctaacctccggccagctgttctcc
 P  T  S  A  V  T  A  F  L  E  L  D  L  T  S  G  Q  L  F  S
cccggaaccccgttgtcctactcagaacccggctttgtgaaattgccccgcgcagatcgt
 P  G  T  P  L  S  Y  S  E  P  G  F  V  K  L  P  R  A  D  R
gagaaggtgggggaacaggcattcgccgatcttcaatcgctcaaaaagttagtcgccgag
 E  K  V  G  E  Q  A  F  A  D  L  Q  S  L  K  K  L  V  A  E
gtgaaggcctatcgcaagacagcgagggcgaatgggaacaccacgctcgctgaccggtgc
 V  K  A  Y  R  K  T  A  R  A  N  G  N  T  T  L  A  D  R  C
acagcacagctcataagactcgcccaagccctcgagagcctgaccgcctga
 T  A  Q  L  I  R  L  A  Q  A  L  E  S  L  T  A  -
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Fig. 3. *LipHim1* gene with translated nucleotide sequence. (A) Amino acid alignment of or lipase with *A. hydrophila* lipase. Only aligned regions are shown. (B) Translated sequence of cloned novel lipase from metagenomic DNA. Rare amino acid codons are underlined by bold line.

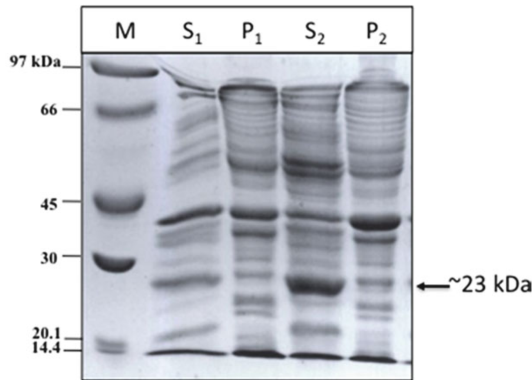

Fig. 4. SDS-PAGE analysis RIL cells over-expressing LipHim1 lipase from the pETLip plasmid. Pellet and soluble protein lysates were prepared and subjected to SDS-PAGE analysis. S indicates soluble fraction and P indicates pellet fraction.

Fig. 6. Lipolytic activity of culture supernatant of lipase over-expressed in BL21 (DE3) RIL cells carrying pETLip. Bacterial supernatant (soluble fraction) containing over-expressed lipase was added to the wells in the agar plates containing the Tween 60 as a substrate for lipase. Numbers indicate wells containing different concentrations of the soluble fractions.

pBluescript KS vector (pBKS-Lip). pBKS-Lip plasmid was isolated from several clones and subjected to DNA sequencing using M13 forward and reverse primers (Table 2). One plasmid with right orientation of metagenomic DNA was selected and further used for cloning of putative lipase gene.

Identification of a putative lipase gene

DNA sequencing was performed to identify the lipase gene using primers M13F, LFP2, PPF, PPR, and M13R (Table 2) and pBKS-Lip as a DNA template. Location of binding sites of primer used for DNA sequencing is indicated in Fig. 2. The complete 2.8 kb of DNA sequence was aligned and analyzed as described in materials and methods. ORF-finder (NCBI) analysis of the sequence revealed that 2.8 kb DNA fragment comprised of three open reading frames with 196, 270, and 397 amino acids (aa), respectively (HM 130054). BLASTX analysis indicated that the 591 bp DNA fragment encoding for ORF with 196 aa have similarity with known extracellular lipase of *A. hydrophila*. 196 aa ORF had 32% similarity to an extracellular lipase of *A. hydrophila* at the amino acid sequence level and the similarity was confined only to N-terminal region of the *A. hydrophila* lipase (Fig. 3A).

Protein expression and analysis of putative lipase gene

To further characterize the putative extracellular lipase, 591 bp DNA fragment encoding 196 aa open reading frame was PCR amplified by using specific primer LFNd1 and LRS1 and cloned into pET28b expression vector, pET-Lip, as described in materials and methods. We tried to over express the lipase protein in BL21 (DE3) and Rosetta 2 (DE3) bacterial expression system. Unfortunately, both the strains expressed negligible or very less quantity of proteins, making it difficult to purify this lipase protein. Further, careful analysis of the sequence of 591 bp putative lipase gene revealed the presence of rare codons (Fig. 3B, underlined codons). In order to see whether low expression of protein was indeed due to rare codons in the sequence, we expressed this protein in BL21 (DE3) codon plus RIL cells (Stratagene), which can express genes with such rare codons. We were successful in expressing lipase protein in RIL cells. Expression of His-tagged lipase was detected on SDS-PAGE in cells induced with IPTG (Fig. 4, Lane S2) and confirmed by Western analysis using anti-His antibodies against His-tagged lipase protein (Fig. 5).

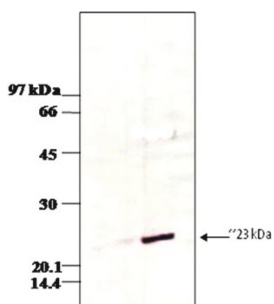

Fig. 5. Western analysis for identification of his-tagged LipHim1 using Anti-His Antibody. Pellet and soluble fractions expressing extracellular lipase were subjected to SDS-PAGE analysis. Immunoblotting was carried out by using anti 6x His tag antibody.

Lipolytic activity of extra-cellular lipase LipHim1

Extra cellular lipase activity of LipHim1 was measured as described in 'Materials and Methods'. Culture supernatant of LipHim1 over expressing RIL cells was added into the holes made on LB agar plates containing Tween 60. The turbidity appeared around the holes indicated the presence of lipase in the culture supernatant (Fig. 6). Increase in area of turbidity with increase in the amount of culture supernatant indicated that LipHim1 is an extracellular lipase.

Discussion

Several novel lipases or esterases are isolated using metagenomic libraries from thermal environments (Rhee *et al.*, 2005), or saline lake (Rees *et al.*, 2003), or forest soil (Lee *et al.*, 2004), or lipases from field soil (Henne *et al.*, 2000; Rondon *et al.*, 2000) or drinking water (Voget *et al.*, 2003). Different substrates have been used to assess the lipase/esterase activity of isolated putative lipases. In this study, we used Tween 60 as a substrate for detecting the lipase/esterase activity. Tweens (fatty acid esters of polyoxyethylene sorbitan) are the most widely used substrates for the detection of lipolytic microorganisms in agar media (Emanuilova *et al.*, 1993; Ploua *et al.*, 1998), since chromogenic and fluorogenic substrates are not stable during the long incubation times required (Macrae, 1983).

In this report, we have identified and characterized a novel extracellular lipase LipHim1, which does not seem to associate

with any family of esterases/lipases according to the classification suggested by Arpigny and Jaeger (1999). However, the sequence of the novel *LipHim1* gene showed only 32% similarly to *A. hydrophila* extracellular lipase gene that belongs to II GDSL family of lipases. In addition, LipHim1 did not exhibit characteristic Gly-Asp-Ser-(Leu) [GDS(L)] motif containing the active-site serine residue. Therefore, we could not assign the identified novel lipase into any of existing lipase group/family. Since the isolated lipase was from metagenomic library, non-bacterial origin of lipase could not be ruled out. Future studies should include characterization of LipHim1 lipase and its industrial usage.

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Competing Interests

The authors declare that there are no competing interests.

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