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Cloning, Expression, and Purification of a Lipase from Psychrotrophic *Pseudomonas mandelii*

Junsung Kim and Chang Woo Lee*

Department of Biomedical Science, Daegu University, Gyeongsan 712-714, Korea

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A gene encoding a lipase, *lipT*, was cloned from the psychrotrophic bacterium *Pseudomonas mandelii* and sequenced. An open reading frame of 1,686 bp was found that encodes a polypeptide consisting of 562 amino acid residues. Sequence analysis revealed a Gly-His-Ser-Leu-Gly sequence, which matches the consensus Gly-X-Ser-X-Gly motif conserved among lipolytic enzymes. The recombinant LipT protein was predominantly expressed as inclusion bodies in *Escherichia coli* and subsequently purified by nickel-chelate affinity chromatography. A small fraction of LipT was refolded, and the subsequent LipT exhibited substrate preferences for *p*-nitrophenyl butyrate (C4) and *p*-nitrophenyl octanoate (C8).

Key words : Lipase, *Pseudomonas fluorescens*, *Pseudomonas mandelii*, Psychrotrophic Bacterium

Introduction

Lipases (EC 3.1.1.3) from cold-adapted bacteria play important roles in industrial applications due to their high enzymatic activity at low temperatures [9]. These applications include the use of immobilized lipases as additives in low-temperature laundry detergent formulations, the treatment of chilled dairy product in the food industry, and as catalysts for developing new therapeutic agents in the pharmaceutical industry [9,18].

Lipases belong to the α/β-hydrolase superfamily and consist of a single domain molecule [8]. Lipases act on esters of long-chain fatty acids which are insoluble in water, whereas esterases act on esters of short-chain fatty acids which are water soluble [3]. The lipase active site contains a catalytic triad consisting of serine, histidine, and aspartate residues [8]. Although lipases share a common catalytic mechanism and structure, they show low levels of sequence similarity at the amino acid level [6].

In this study, we utilized the genome sequence of *Pseudomonas fluorescens* Pf0-1 [17], to which the psychrotrophic bacterium *Pseudomonas mandelii* showed high homology. A gene coding for a lipase, *lipT*, was cloned from *P. mandelii* JR-1 using primers based on the non-coding region sequences surrounding the *P. fluorescens* Pf0-1 lipase gene (UniProt ID: Q3KCS9). The recombinant LipT protein, which was mostly expressed in *E. coli* BL21 (DE3) as inclusion bodies, was purified using nickel-chelate affinity chromatography. After refolding, LipT exhibited substrate preferences for *p*-nitrophenyl butyrate (PNPB) and *p*-nitrophenyl octanoate (PNPO).

***Corresponding author**
Tel : +82-53-850-6464, Fax : +82-53-850-6469
E-mail : leec@daegu.ac.kr

Materials and Methods

Materials

The TA cloning vector was purchased from Enzynomics (Korea). The pET28a expression vector was purchased from Novagen (USA). The HisTrap FF column was purchased from GE Healthcare (USA) and the esters for *p*-Nitrophenyl were purchased from Sigma (USA). All other reagents were obtained from Sigma unless noted otherwise.

Strain isolation and identification

P. mandelii JR-1 was isolated from natural mineral waters collected in Gyeongsan, Korea. Gram staining was performed as described previously [12]. The 16S rRNA sequencing was carried out at Genotech (Korea). The 16S rRNA sequence analysis of the isolated bacterium was carried out using an EzTaxon server [4].

Plate assay for lipase

Rhodamine B agar plates were prepared as described previously [10]. Holes of 3 mm diameter were punched into the agar and the cavities formed were filled with either 10 μl of bacterial culture or cell-free culture supernatant. Plates were incubated overnight at 25°C, exposed to UV irradiation (350 nm), and then photographed.

Gene cloning of *lipT*

The *lipT* gene was cloned from *P. mandelii* JR-1 by polymerase chain reaction (PCR) in two steps. First, primers were designed based on the non-coding region sequences surrounding the lipase (Q3KCS9) in the *P. fluorescens* Pf0-1 genome. The forward primer was 5′-GACCACGGTGT GGGCTTGAC-3′ and the reverse primer was 5′-GCTCACAACCAGAACGCCCC-3′. The resulting PCR product was subcloned into a TA vector and sequenced. Second, the gene for *lipT* was amplified from the TA vector and subcloned into a pET28a vector. The forward primer used was 5′-GAGAGAtctagaAAGGAGATATAC**ATGGGA CTGTTTGATTAC**-3′ (Xba I site in small letters, ribosome binding site sequence underlined and the 5′-end region of *lipT* in bold face type). The reverse primer used was 5′-GCGGCCGCaagctt**CGCAAACGTGATGCCTG**-3′ (Hind III site in small letters and the 3′-end region of *lipT* in bold face type). A linker and His_6 sequence (KLAAALEHHH HHH), which comes from a pET28a vector, were located on the C-terminus of LipT. The construct was confirmed by DNA sequencing.

Sequence analysis

A homology search was performed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple sequence alignments were performed using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Expression and purification of LipT

The gene for LipT with a C-terminal His_6 sequence was transformed into *E. coli* BL21 (DE3). A single colony grown on an LB/kanamycin plate was selected for additional overnight growth at 37°C, followed by inoculation into a 250 ml LB/kanamycin broth. At the mid-log phase (OD_{600nm}= 0.6~0.8) the growth temperature was lowered to 30°C. After addition of 1 mM IPTG, the cells were grown for 4 more hours. The cells were harvested at 10,000× *g* for 5 min. The pellet was resuspended in Buffer A (20 mM Tris · Cl, 0. 1 M NaCl, 5% glycerol, pH 8.0) followed by sonication at 4°C. After centrifugation at 12,000× *g* for 10 min, the pellet was resuspended in Buffer A with 8 M urea. The imidazole concentration was adjusted to 5 mM in preparation for purification using nickel-chelate affinity column chromatography. LipT was purified on an AKTA Explorer system (GE Healthcare) with a 1-ml HisTrap column using Buffer B (20 mM Tris · Cl, 0.1 M NaCl, 8 M urea, 70 mM imidazole, 5% glycerol, pH 8.0). All purification steps were carried out at 4°C. The purified enzymes were frozen in N_2 and stored at -80°C.

Enzyme assay

The substrate specificity of LipT for 0.4 mM *p*-nitrophenyl esters (C2 to C16) was measured in reaction buffer (100 mM Tris · Cl, 100 mM NaCl, 0.3% Triton X-100, pH 8.5). The accumulation of *p*-nitrophenol was measured using a Shimadzu UV-160 spectrophotometer at 400 nm for 5 min at 25°C.

Nucleotide sequence accession number

The nucleotide sequence of the *lipT* gene from *P. mandelii* JR-1 has been deposited in GenBank under accession number JQ284021.

Results and Discussion

Strain identification and phylogenetic analysis

Taxonomical studies based on the 16S rRNA sequence revealed homology to the genus *Pseudomonas* (Fig. 1). Analysis using the EzTaxon server showed it possessed the highest level of similarity to *Pseudomonas mandelii* (100%), followed by *Pseudomonas cannabina* (99.53%) and *Pseudomonas tremae* (99.46%). A BLAST search of the NCBI database also confirmed high degrees of similarity to *P. mandelii* strains. *P. mandelii* is classified as belonging in the *P. mandelii* subgroup of the *Pseudomonas fluorescens* group [13].

Optimum growth temperature

As demonstrated by Gratia et al. [7], *P. mandelii* grew at 4°C but did not grow at 37°C. Although *P. mandelii* JR-1 is a psychrotrophic bacterium, its optimum growth rate was at 25~30°C (data not shown).

Investigation of lipase activity

To investigate *P. mandelii* lipase activity, we utilized agar plates containing olive oil and rhodamine B as previously demonstrated by Kouker and Jaeger [10]. Free fatty acids released from the olive oil formed colored complexes with rhodamine B, which is a basic dye. Upon UV irradiation, orange fluorescence was observed for the *P. mandelii* culture on the rhodamine B agar plates, whereas no fluorescence was observed for *E. coli* (Fig. 2). The *P. mandelii* culture supernatant also revealed orange fluorescence (data not

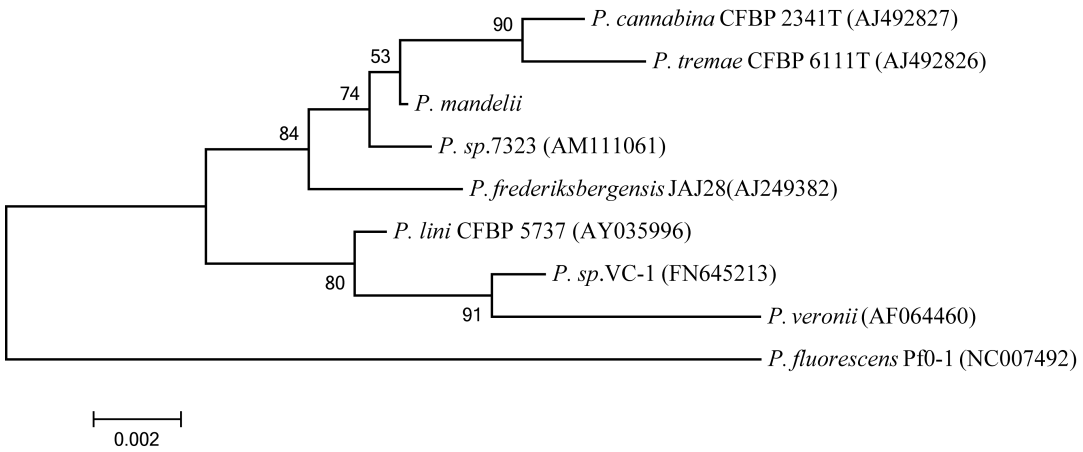

Fig. 1. Phylogenetic tree based on the 16S rRNA gene sequence of *P. mandelii* JR-1. *P. mandelii* belongs to the *P. mandelii* subgroup of the *P. fluorescens* group [13].

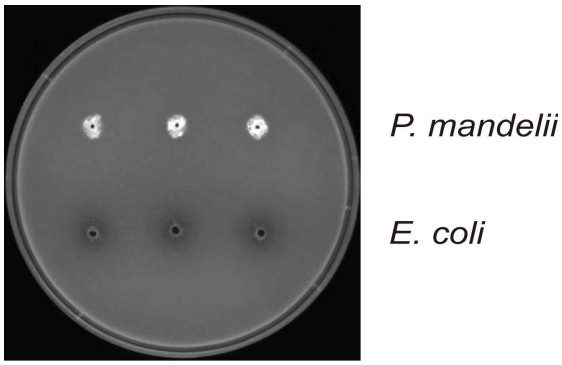

Fig. 2. Identification of lipase activity on a rhodamine B agar plate. After overnight incubation at 25°C, plates were exposed to UV irradiation at 350 nm.

shown). Our data indicated that *P. mandelii* produced lipase activity, of which, some was exhibited by extracellular lipases.

Cloning of the *lipT* gene

Generally, a bacterium expresses several genes of lipases and esterases, possibly reflecting a wide range of substrate specificities. We used two lipase-prospecting primers (OXF1-ACR1 and OXF1-ACR3), as reported by Bell et al. [2], to clone lipase genes from *P. mandelii* JR-1 (data not shown). Although sequencing of the PCR products did not locate a lipase, a BLAST search revealed that the sequences had the highest degree of homology to those of *P. fluorescens* Pf0-1 from among the repository of completed microbial genomes (data not shown). Thus, the PCR primers were designed based on the non-coding region sequences surrounding four *P. fluorescence* pf0-1 lipases, found as lipase in the UniProt database (UniProt ID: Q3KBP5, Q3KCS8, Q3KCS9, and Q3KIU1). A PCR product was amplified from the non-coding region sequences surrounding a lipase (Q3KCS9), subcloned into a TA vector, and then sequenced. A 1,689-bp *lipT* gene amplified from the TA vector was subcloned into a pET28 vector. Surprisingly, both the *lipT* and the *P. fluorescens* Pf0-1 lipases had the same 1,689-bp sequence length.

Sequence analysis

The deduced LipT amino acid sequence comprised 562 amino acids residues. LipT contained a Gly-His-Ser-Leu-Gly sequence, a motif characteristic of the serine lipase family (Gly-X-Ser-X-Gly) [16] (Fig. 3). Sequence analysis also showed that LipT contains six motifs (Gly-Gly-X-Gly-X-Asp or Gly-X-X-Gly-X-Asp) which function as the C-terminal signal for identification by ABC transporters [1,5] (Fig. 3). LipT showed 83% sequence similarity at the amino acid level to the *P. fluorescens* Pf0-1 lipase (Q3KCS9).

Expression and purification of LipT

An entire open reading frame of *lipT* with a C-terminal His_6 residues was constructed on a pET28a vector. LipT expression in *E. coli* BL21 (DE3) increased in a time-dependent manner (Fig. 4A), mainly resulting in inclusion body formation. Purification of LipT was carried out with 8 M urea using a 1-ml HisTrap column. LipT was eluted at 70 mM imidazole, as shown in Fig. 4B, suggesting that it bound weakly to the nickel resin. LipT was refolded by reducing the urea concentrations, but only a small fraction of LipT was refolded such that it resulted in lipase activity.

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LipT      MGLFDYKNADGKALYSDAIALTLYAYTPTGQPLPATAWAPIGAKQLGYQGKVGAQGTFYG 60
Q3KCS9    MGLFDYKNADGKALYSDAIALTLYAYTPTGQALPGTGWKPIGATALGYQGKVGVQGTFFG 60
          ********************************.**.*.* ****. ********.****:*

LipT      EKDGFTSAEAEVLGKYDTAGKLIGIGIAFRGTGGLGYSDTFGDMKNNLLAAVGPVDYATN 120
Q3KCS9    EKDGFTSAEAEVLGKYDAAGKLIGIGVAFRGTGGLGYSDTFGDMKNNLLAAIGPSDYATQ 120
          ******************:********:*************************:** ****:

LipT      YAKNAFDNLLKDVAAFSIAHGLSARDVMVSGHSLGGLGVNSLAELSGNNWGGFYKDANYI 180
Q3KCS9    YAKNAFDNLLKSVAAFAVAHGIAAKDVLVSGHSLGGLGVNSVAELSASNWGGFFKDANYI 180
          ***********.****::***::*:**:*************:****..*****:******

LipT      AFASPTQSATGNNVLNIGYENDPVFRVLDGTTFSSGSLGKHDGHQDSATNNIVNFNDQYA 240
Q3KCS9    SFASPTQSSTGTNVLNIGYENDPVFRVLDGTTFSTASMGKHDKPHDSTTDNIVNFNDNYA 240
          :*******:**.**********************.*:**** :**:*:*******:**

LipT      STAQNLVPFSILNPLNWSAHGSLGYADGLNRVIDSRFYDLTDKDSTLIVSNLAESSRGTT 300
Q3KCS9    STAQNLVPFSIANPLNWSAHSSLGYADGLNRVIASKFYGLTHKDSTIIVSNLEDASRGKT 300
          *********** ********.************ *:**.**.****:***** ::***.*

LipT      WVEDLGRSGEPHTGSTFIIGTDSDDLLKGGAGNDFIEGRDGNDRLRDDGGYNLLLGGKGS 360
Q3KCS9    WVEDLGRSGEPHTGSTFIIGTDSGDWLKGGAGNDFLEGLGGDDRFRDDGGFNILLGGQGH 360
          **********************.* *********:** .*:**:*****:*:****:*

LipT      NTFELQKPLQNFSFANDGDGTLYVRDAYGGISMTRDIGSLVSKESGSWWGSKEVTYSVTA 420
Q3KCS9    NTFELQKPLQNFSFANDGDGTLYVRDAYGGISMTRDIGALVSKESGSWWGSKEITWTVTA 420
          ************************************:**************:*::***

LipT      NGLLNGSELTHYNHSLNGDAYGNTLAASVDGDWLFGHAGDDLLRSDKSQVTFVGGAGNDL 480
Q3KCS9    KGLANGAELTQYNHSLSGGAMGDTLKATADGDWLFGLGGNDHLQSDKAHVTFVGGAGNDV 480
          :** **:***:*****.*.* *:** *:.******* .*:* *:***::**********:

LipT      MQASGGHNTFLFSGAFGFDAINGYQGNDKLVFLGVQGAGQGYDYKQHASQSGHDTVLKVG 540
Q3KCS9    MSAVGGNNTFLFSGAFGFDAINGYQGSDKLVFMGVEGAGQGYDYKQHASQSGSDTVLKIG 540
          *.* **:******************.*****:**:*************** *****:*

LipT      DFAVTLVGVGLDSLSASGITFA 562
Q3KCS9    DFAVTLIGVGVANLSDSSFVFA 562
          ******:***: .** *.:.**
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Fig. 3. Multiple amino acid sequence alignment. The amino acid sequence of LipT was compared with the *P. fluorescens* Pf0-1 lipase (Q3KCS9). The GXSXG motif (purple) of serine enzymes and the GGXGXD and GXXGXD motifs (cyan) for ABC transporters are denoted, respectively.

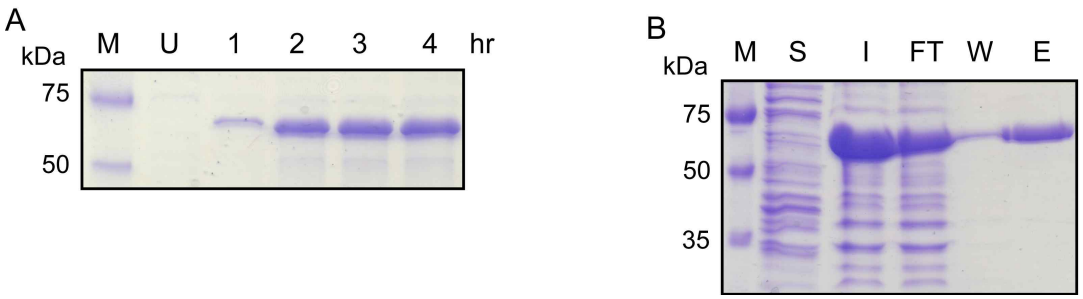

Fig. 4. Expression and purification of LipT. (A) LipT was expressed in *E. coli* BL21 (DE3) upon 1 mM IPTG induction. U, uninduced. (B) Purification of LipT using nickel-chelate affinity chromatography. M, molecular weight marker; Lane S, soluble fraction after sonication; Lane I, Input from inclusion bodies; Lane FT, Flow-through; Lane W, Wash buffer (5 mM imidazole); Lane E, Elution buffer (70 mM imidazole). The calculated molecular weight of LipT was 59,055 Da and it migrated on a SDS gel as a 64 kDa protein.

Substrate specificity of LipT

Of all the *p*-nitrophenyl esters (C2 to C16) tested, LipT exhibited maximal hydrolysis with PNPB (C4) (Fig. 5). The hydrolysis of PNPO (C8) was approximately 71% of that observed for PNPB. LipT showed no enzymatic activity for other *p*-nitrophenyl esters (C2 and C12-16).

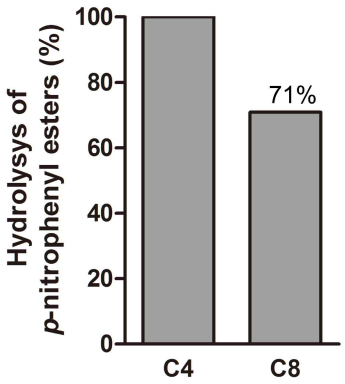

Fig. 5. Hydrolysis of *p*-nitrophenyl esters by LipT. The hydrolysis of the *p*-nitrophenyl esters (C2 to C16) was expressed as a percentage comparison to PNPB. There was no activity observed with C2 and C12 to C16 substrates. 100%=1.4 μM/min.

A few other lipases demonstrated substrate preferences for medium-chain fatty acids which were similar to that of LipT. A lipase from a cold-adapted *Psychrobacter* sp. exhibited the highest hydrolytic activity with *p*-nitrophenyl caproate (C6), followed by PNPB [14]. Medium-chain acyl group *p*-nitrophenyl esters were also good substrates for psychrotrophic *Pseudomonas* sp. KB700A lipase (C10>C6>C4) [15]. A lipase from psychrotrophic *Aeromonas* sp. LPB4 favored substrates containing medium chain acyl groups (C3 to C10) [11].

In conclusion, *lipT*, a gene coding for a lipase, was cloned from the psychrotrophic bacterium *P. mandelii* JR-1 and sequenced. The recombinant LipT protein was expressed in *E. coli* BL21 (DE3), mainly as inclusion bodies. LipT demonstrated substrate preferences for esters of medium-chain fatty acids (C4 and C8).

Acknowledgement

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초록 : *Pseudomonas mandelii*의 lipase 유전자 클로닝, 발현 및 정제

김준성 · 이창우*
(대구대학교 의생명과학과)

내냉성 세균인 *Pseudomonas mandelii*로부터 lipase 유전자(lipT)를 클로닝하고 염기서열을 분석하였다. 열린해독틀(open reading frame)은 1,686 bp로 구성되어 있고, 562개의 아미노산을 코딩한다. 서열분석 결과 많은 세린 효소에서 발견되는 Gly-X-Ser-X-Gly 모티프가 존재한다(Gly-His-Ser-Leu-Gly). 재조합 LipT 단백질은 대장균에서 주로 inclusion body 형태로 발현되었다. 니켈 친화성 크로마토그라피 방법으로 LipT 단백질을 분리하였으며 소량의 LipT 단백질이 refold 되었다. 이 효소는 *p*-nitrophenyl butyrate (C4)과 *p*-nitrophenyl octanoate (C8)에 대해 기질 특이성을 나타내었다.