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# Novel *S*-enantioselective lipase TALipB from *Trichosporon asahii* MSR54: Heterologous expression, characterization, conformational stability and homology modeling

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ABSTRACT

A novel lipase encoding gene, *TALipB* from *Trichosporon asahii* MSR54 was heterologously expressed in *Escherichia coli* using three vectors, pET22b, pET28a & pEZZ18. The three recombinant proteins, *viz.* C-hexahistidine fused HLipB, N and C-hexahistidine fused HLipBH and ZZ-fused ZZLipB were purified using affinity chromatography. All the three enzymes were mid to long fatty acyl chain selective on *p*-NP esters and *S*-enantioselective irrespective of tags. HLipB had lowest activation energy (3.5 Kcal $mol^{-1}$) and highest catalytic efficiency (254 $mM^{-1}$ $min^{-1}$) on *p*-NP caprate followed by HLipBH and ZZLipB. However, ZZLipB demonstrated best pH stability (pH 6–10), thermostability ($t_{1/2}$ of 50 min at 70 °C) and stability toward the denaturant Guanidium chloride (300 mM). Far-UV CD and fluorescence studies confirmed the role of N-terminal ZZ-tag in stabilizing the protein by altering its secondary and tertiary structures. All the three proteins were thiol activated. ZZLipB required higher concentration of β-mercaptoethanol as compared to the other two proteins to attain similar velocity. This indicated the involvement of additional disulfide bonds in its conformational stability. *In silico* analysis suggested low sequence identity of the enzyme with the available database but a close structural homology with *Candida antarctica* lipase B (CALB) was revealed by PHYRE$^2$. MULTALIN with CALB predicted the active site residues (Ser137–Asp228–His261) which were confirmed by superimposition and site directed mutagenesis.



## 1. Introduction

Lipases, also known as triacylglycerol hydrolases (E.C. 3.1.1.3), are serine hydrolases that catalyze the hydrolysis and synthesis of short to long-chain acylglycerols, fatty acid esters and triglycerides. Lipases are important industrial enzymes due to their desirable properties such as high enantioselectivity, regioselectivity for a broad range of substrate and high stability to pH, temperature and inorganic solvents [1].

Enantioselective lipases are indispensable in the field of pharmaceuticals for the chiral resolution of drugs. Bacterial and yeast lipases are commonly exploited for this application with yeast lipases holding a definite edge due to their high enantiomeric excess. Despite a huge demand, very few yeasts lipases are commercially available, most notable being the lipases from *Candida* species such as *Candida rugosa* and *Candida antarctica* [2]. Many researchers have focused on this aspect and efforts are underway to improve enantioselective behavior of present lipases or discover novel yeast lipases that can fulfill this large void.

In this respect, our group has isolated a basidiomycetous yeast, *Trichosporon asahii* strain MSR54 (MTCC No. 9459), from petroleum sludge [3]. This yeast produces three extracellular lipases, TALipA, B and C. Of these, we have previously reported detailed study on TALipA and C, both of which were found to be enantioselective [4]. The current study focusses on the third lipase, TALipB. The sequence of TALipB from *T. asahii* MSR54 has been submitted in NCBI (gene ID KC732451.1) by our group. The gene was intron-free and encoded a protein of 33 kDa. Here, we report for the first time the heterologous cloning and expression of TALipB in *Escherichia coli* using different host-vector combination. The recombinant enzymes were purified by affinity chromatography and further characterized in terms of their functional and conformational characteristics.

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## 2. Material methods

### 2.1. Cloning and expression

Primers for TALipB encoding gene were designed (Table S1) from the gene sequence of TALipB submitted by our group at NCBI (gene ID KC732451.1). The gene was amplified from the genomic DNA of *T. asahii* MSR54 (MTCC No. 9459), available in our laboratory. The amplified product was then cloned into pGEMT-Easy vector.

The gene was sub-cloned into three vectors *viz.* pET22b, pET28a and pEZZ18. The recombinant vectors pET22b-*TALipB* and pET28a-*TALipB* were transformed into *E. coli* BL21 (DE3) while pEZZ18-*TALipB* was transformed into *E. coli* HB101. Cells were cultivated at 37 °C/200 rpm for pET22b and pET28a and 37 °C/300 rpm for pEZZ18 in 50 ml LB medium containing 100 μg/ml ampicillin. Transformed cells containing pET22b-*TALipB* and pET28a-*TALipB* were induced with 0.4 mM IPTG at $OD_{600}$ of 0.6 and further incubated at 37 °C for 6 h. Cells containing pEZZ18-*TALipB* were incubated at 37 °C for 18 h without induction. The active lipase enzymes from the three vectors pET22b (C-terminal His-tag), pET28a (N and C-terminal His- tag) and pEZZ18 (N-terminal ZZ-tag) were named as HLipB, HLipBH and ZZLipB, respectively.

### 2.2. Enzyme extraction and purification

After 6 h of induction, cells were harvested by centrifugation at 10,000 × *g*/10 min and washed two times with 50 mM phosphate buffer (pH 7.5). The cell pellet was suspended in the same buffer and sonicated using 750-W/20 kHz High Intensity Ultrasonic sonicator, to release intracellular and periplasmic proteins. The cell lysate was centrifuged at 10,000 × *g* for 20 min to remove cell debris and the clear supernatant obtained was used for purification.

For purification of the His-tagged proteins HLipB and HLipBH, 3 ml of $Ni^{2+}$-Nitrilotriacetate (NTA) resin (Qiagen, CA, USA) was used according to manufacturer protocol. Elution of the tagged protein was done with a linear imidazole gradient (100 ml of 0–300 mM in 50 mM phosphate buffer, pH 7.5, 10 mM NaCl).

For ZZLipB, after 24 h of incubation at 37 °C/300 rpm, cells were harvested at 10,000 g/10 min. This cell free supernatant (1000 ml) was precipitated by 80% ammonium sulphate saturation while maintaining a constant pH of 7.5. The precipitate obtained was resuspended in 50 ml of 50 mM phosphate buffer (pH 7.5) and dialyzed with 10 mM phosphate buffer (pH 7.5) for 12 h. The dialyzed protein was then purified using IgG Sepharose column (GE Healthcare) and purified using the manufacturer's protocol with the difference being that protein elution was done in 50% 1–4 Dioxane [4].

The purity of the three purified proteins was checked by 12% SDS-PAGE [5] and quantified by protein quantification kit (Fluka). The purified proteins were used for further studies.

### 2.3. Lipase assay

Lipase activity was assayed spectrophotometrically using *p*-nitrophenyl caprate (*p*-NPC) as the substrate [6]. One enzyme unit of lipase is defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per minute under the standard assay conditions.

### 2.4. Effect of pH and temperature

For pH optima, the substrate was prepared in different buffers (pH 5.0–11.0). Optimum temperature was evaluated using *p*-nitrophenyl caprate at a wide range of temperatures (30–90 °C). pH stability of the enzymes was determined by pre-incubating the enzyme in buffers of different pH values at room temperature for 120 min and measuring the residual activity at optimum pH and temperature. Thermostability of the enzymes was investigated by incubating the enzyme at different temperatures (40–70 °C) and measuring the residual activity after every 15 min upto 1 h. The rate of enzyme deactivation, $k_d$ was estimated by linear regression analysis of the natural logarithm of residual activity *versus* treatment time. The half-life ($t_{1/2}$) for lipase thermal denaturation was calculated as:

$$t_{1/2} = \frac{1n(2)}{k_d}$$

where $k_d$ = slope.

### 2.5. Substrate specificity of lipase

Substrate specificity of the lipases was determined spectrophotometrically using *p*-nitrophenyl esters (C4–C18) and by titrimetric method on oils (10% v/v) [7].

### 2.6. Enzyme kinetics

To study the kinetic parameters, lipase assay was performed with 0.2–2.0 mM concentrations of *p*-NP caprate at different temperatures (20–70 °C). Lineweaver–Burk plot and Michaelis–Menten equation were used for calculating $K_m$, $V_{max}$, $K_{cat}$ and $K_{cat}/K_m$.

Activation energy ($E_a$) was calculated by plotting log $V_{max}$ *versus* $1/T$ where $T$ is given in °K. The plot has a slope of $(\log V_{max2} - \log V_{max1}/T_2 - T_1)$ where the slope is equal to $-E_a/2.3$ ($R$) and $R$ is the gas constant (1.98 cal $K^{-1}$ $mol^{-1}$).

### 2.7. Effect of metal ions, inhibitors and solvents

Effect of various divalent metal ions (10 mM), inhibitors (10 mM) and solvents (100%) was studied by pre-incubating 100 mg of the enzymes for 1 h and residual activity was determined (4).

### 2.8. Circular dichroism spectroscopy

CD spectroscopy is an important technique in structural biochemistry for determining the structure of proteins. 0.5 mg/ml of proteins was used for the CD spectral analysis (JASCO corp, Japan). The change in the secondary structure of protein was monitored in the far-UV region between 190 and 260 nm.

For thermal kinetics, the CD-spectra were recorded by incubating the enzyme at different temperatures (30–70 °C) for 5 min at 10 °C increment. The incubation temperature was increased at the rate of 2 °C/min and spectra were carried out at a 20 nm/min scan speed with a response time of 1 s and 2 nm bandwidth. Any deviations from the standard protocol have been mentioned in the respective experiments.

### 2.9. Fluorescence spectroscopy

Intrinsic fluorescence emission spectra of lipases were monitored using a Perkin-Elmer fluorescence spectrophotometer and a scanning emission monochromator using the PiStar software. 0.5 mg/ml protein was used to measure the fluorescence intensity. Excitation was done at 295 nm and emission was registered at 300–400 nm, using a 5 nm bandwidth in both the excitation and emission paths. In case of thermal kinetics, fluorescence intensity was recorded after pre-incubating the enzyme in the temperature cell of the instrument for 30 min at different temperatures (40–70 °C). For the remaining studies, the enzymes were subjected to the desired conditions and used for measuring the fluorescence intensity. Any deviations from the standard protocol have been mentioned in the respective experiments.