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# Combinatorial reshaping of a lipase structure for thermostability: Additive role of surface stabilizing single point mutations

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ABSTRACT

Thermostable lipases are of high priority for industrial applications. In the present study, targeted improvement of the thermostability of a lipase from metagenomic origin was examined by using a combinatorial protein engineering approach exploring additive effects of single amino acid substitutions. A variant (LipR5) was generated after combination of two thermostabilizing mutations (R214C & N355K). Thermostability of the variant enzyme was analyzed by half-life measurement and circular dichroism (CD). To assess whether catalytic properties were affected by mutation, the optimal reaction conditions were determined. The protein LipR5, displayed optimum activity at 50 °C and pH 8.0. It showed two fold enhancement in thermostability (at 60 °C) as compared to LipR3 (R214C) and nearly 168 fold enhancement as compared to parent enzyme (LipR1). Circular dichroism and fluorescence study suggest that the protein structure had become more rigid and stable to denaturation. Study of 3D model suggested that Lys355 was involved in formation of a Hydrogen bond with OE1 of Glu284. Lys355 was also making salt bridge with OE2 of Glu284.



## 1. Introduction

Protein stability is often a limiting factor in the development of commercial proteins and biopharmaceuticals, as well as for biochemical and structural studies [1]. Unfortunately, identifying stabilizing mutations is not trivial since most are neutral or deleterious. Biocatalysts like lipases often need to be robust to meet the harsh reaction conditions [2]. They can catalyze reactions of insoluble substrates at the lipid–water interface, preserving their catalytic activity in organic solvents [3]. This makes lipases the most powerful tool for catalyzing not only hydrolysis, but also various reverse reactions such as esterification, transesterification, aminolysis, or thiotransesterifications in anhydrous organic solvents [4–6]. They display important advantages over classical catalysts, as they can catalyze reactions with reduced side products, lowered waste treatment costs, and under mild temperature and pressure conditions [7]. Accordingly, the use of lipases holds a great promise for green and economical process chemistry [8,9].

However, performance of a lipase is not always sufficient for an industrial application [9] and most enzymes have sub-optimal properties for processing conditions [10]. In order to improve enzyme-mediated process efficiency we need to modify the enzymes suitable for a defined industrial process [9,10]. Knowledge based protein design as well directed evolution based approaches have been used to achieve this goal [11–17].

Thermal stability is a major requirement for commercial enzymes, being critical for industrial applications, as thermal denaturation is a common cause of enzyme inactivation [18]. In effort to meet the industrial demands, many lipases have been engineered to enhance their thermostability, including *Candida antarctica* lipase B [19,20], *Rhizomucor miehei* lipase [21], or *Bacillus subtilis* lipase [22].

In our lab we are actively engaged in the directed evolution of lipases for the last decade [23–29]. Recently, we had isolated lipases from metagenomic sources namely LipR1 and JkP01 [28,29] and evolved them successfully for thermostability as well as enzyme activity. Among them the proteins LipR3 (R214C) and LipM1 (N355K) contain single point mutations [29]. Since, these proteins were highly identical (96% sequence identity) and LipM1 was more thermostable than LipR3, therefore we decided to incorporate mutation of LipM1 to LipR3. We were interested to see, whether these mutations (N355K and R214C) will work additively or not. Therefore an attempt was made to mutate this residue in LipR3 by site directed mutagenesis to demonstrate its effect on thermostability. The mutant (LipR5) containing both the mutation was characterized in detail.

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## 2. Materials and methods

### 2.1. Reagents/kits/vectors

All the chemicals and reagents used in the study were of good analytical grade, obtained from Merck (Germany)/Sigma Aldrich (USA)/ HiMedia (India). pGEM-T easy vector used for cloning purpose was purchased from the Promega (USA). pQE30-UA, used as expression vector was purchased from the Quiagen (Germany).

### 2.2. Cloning of LipR5 by site directed mutagenesis

Site directed mutagenesis was carried out by means of QuikChange® XL site-directed mutagenesis kit obtained from stratagene, according to the manufacturer's instructions. Plasmid of LipR3 was used as template with forward and reverse primers for mutagenesis:

Forward primer 5′ TGGGAACGTACAAAGTCGACCATT 3′
Reverse primer 5′ AATGGTCGACTTTGTACGTTCCCA3′

The nicked vector DNA incorporating the desired mutations was then transformed into XL10-Gold®*** ultracompetent cells.

### 2.3. Nucleotide sequence analysis and intracellular cloning

To confirm the mutation, the plasmids from the few selected clones were sequenced using universal M13 forward and reverse primer. The nucleotide sequencing was done by commercial available service provided by Chromous biotech (India). Upon sequence analysis, the mutation N355K was confirmed at the desired position. The open reading frame (ORF) of selected lipase gene was cloned intracellularily in pQE-30UA expression vector using primers designed for intracellular cloning (Forward primer 5′-GGATCCATGGCATCTCGACGC-3′ and Normal reverse primers), and expressed in *Escherichia coli* M15 cells containing pREP4 plasmid as per manufacturer's instructions.

### 2.4. Purification of LipR5

All steps for purification of LipR5 enzyme was carried out at 4 °C (unless otherwise stated) according to the protocol of Kumar et al. (2013) [27]. LipR1 and LipR3 enzymes were also purified simultaneously for comparison.

### 2.5. Enzyme assay

All the enzymatic assays for determination of residual and relative enzyme activities were carried out according to the method of Kumar et al. (2013) [27]. The total enzyme activity was expressed in U and specific activity was expressed as U/mg of protein. One unit of enzyme activity is defined as the amount of enzyme, which librates 1 μ mol of pNP from pNP-laurate per minute under standard assay conditions. The protein concentration was determined at each purification step using the commercially available BCA (Bicinchoninic acid) kit (Banglore-Genei, India). Bovine serum albumin was used as standard and absorbance was recorded at 562 nm.

### 2.6. Biochemical characterization

#### 2.6.1. Polyacrylamide gel electrophoresis

The dialyzed protein was analyzed for purity under denaturing condition on 12% SDS–PAGE gel.

#### 2.6.2. Effect of temperature on enzyme activity

Optimum temperature for the LipR5 lipase was determined by assaying the enzyme activity at different temperature (20–80 °C).

#### 2.6.3. Thermal inactivation of enzyme

Thermal denaturation profile of the enzyme was studied by pre-incubating the enzymes separately, at 55 °C, 60 °C, 65 °C and 70 °C respectively. Enzyme aliquots were taken out at different time intervals, cooled at 4 °C for 15 min followed by enzyme assay. Enzyme without incubation was taken as control (100%). Further, the enzyme activity at the start of the experiment was taken as 100%, and the residual lipase activity after incubation was determined. Reaction mix without enzyme served as blank.

#### 2.6.4. Effect of pH on enzyme activity and stability

Optimum pH for the purified lipase (LipR5) was determined by assaying the enzyme in buffers of different pH (sodium acetate—pH 5.0, sodium phosphate—pH 6.0–8.0, Tris–HCl—pH 9.0, Glycine NaOH—pH 10.0–11.0) at 50 °C and 45 °C respectively. The pH stability of the lipase was determined by pre-incubating the enzyme with 0.05 M buffer of different pH (5.0–11.0) for 1 h at room temperature followed by enzyme assay.

#### 2.6.5. Substrate specificity

Substrates specificity for LipR5 was studied using pNP ester (final concentration 0.2 mM) of following chain length: pNP-acetate (C3), pNP-caprylate (C8), pNP-decanoate (C10), pNP-laurate (C12), pNP-myristate (C14), pNP-palmitate (C16), pNP-stearate (C18) from Sigma (USA) were dissolved in absolute alcohol, and assayed according to standard assay method.

#### 2.6.6. Effect of additives and detergents

Effect of various concentrations (10% v/v) of organic solvents, n-Hexane, Acetone, Toluene, Ethylene glycol, DMSO, Glycerol and Methanol, on enzyme activity of both the enzymes were monitored. The purified enzymes (0.1 ml) were incubated with the solvents (10% v/v organic solvent + Sod. Phosphate buffer) for 1 h at room temperature then residual activity was checked by normal enzyme assay protocol. Effect of different additives (0.1 mM each) such as, diethylpyrocarbonate (DEPC), β-mercaptoethanol (β-ME), Tween 20–80 (1%, v/v), Triton X-100 (1%, v/v), sodium dodecyl sulfate (SDS) (1%, w/v), were studied on enzyme activity. The enzymes were incubated with additives at 37 °C for 5 min before enzyme assay. The reaction mix with respective additives but without enzyme served as blank. The reaction mix without any additives was taken as control (100%).

#### 2.6.7. Inhibition study

We have tested the effect of PMSF (a serine inhibitor) on enzyme activity. PMSF (100 μl), of different concentration was added to the reaction mix (Sodium Phosphate buffer 700 μl + 100 μl enzyme). The reaction mix was incubated, at 50, 55 and 60 °C for 3 min respectively, before the substrate (100 μl) was added. The enzyme assays were performed according to standard assays method.

#### 2.6.8. Kinetic parameter

Enzyme activity as function of substrate concentration (0.01–2 mM) was determined for both enzymes. The Michaelis–Menten constant ($K_m$) and maximum velocity for the reaction ($V_{max}$) with pNP-laurate as substrate, were calculated by Lineweaver–Burk plot. The $k_{cat}$ and $k_{cat}/K_m$ were also calculated for both LipR3 and lipR5, and the results were compared with LipR1.

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