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Engineering of A Lipase towards Thermostability: Studies on Additive Effect of the two Thermo-Stabilising Mutations at Protein Surface

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Abstract

In this study we have showed combined effect of two single point mutations S311C (LipR2) and R214C (LipR3) on the protein stability and overall change in biochemical properties. We found that both of these mutations are near the surface and individually enhanced the thermal stability of the protein (T1/2 for S311C=4.5 h & R214C=7 h at 60°C). But, their combined effect was not additive on thermostability. T1/2 of double mutant (LipR2 + LipR3) was 4 h at 60°C. Circular dichroism (CD) and fluorescence studies also supported our findings. Homology modelling studies demonstrated that in double mutant (LipR4) side chain of Cys311 is protruding towards the bulk solvent and is easily available for oxidation of sulfahydril group. This might be the reason for its low thermostability as compared to LipR3. We also observed that, side chains of Cys 214 didn't changed. Here, one of the Cystein (Cys311) is behaving like a hydrophilic residue while the other (Cys 214) is behaving like hydrophobic residue.

Keywords: Lipase; Thermostability; Mutations; Enzyme; Nucleotide sequence

Introduction

The biotechnological potential of hydrolytic enzymes is of special interest because of their extensive use in industries. Among them, lipases are of special interest. Lipase-catalyzed reactions are important because they successfully catalyzes various types of reactions such as interesterification, acidolysis, esterification, alcoholysis, and aminolysis in addition to its hydrolytic activity on triglycerides [1-3].

Regardless of process conditions, thermostability is an important parameter in successful industrial bioprocesses [4,5]. Thermostable lipases are of high priority for industrial applications as they are capable of carrying out diversified reactions at elevated temperatures. Several reasons have been attributed to the greater stability of the thermophilic proteins [6-8]. Among the most prominent ones are better packing, greater hydrophobicity, shortening of loops [9-11] amino acid substitutions within and outside the secondary structures [12,13], decreased occurrence of thermolabile residues, increased hydrogen bonding and salt bridges [14,15]. However, no single traffic rule for correlation of thermostability with particular amino acids can be generalized [16-19].

Therefore search for new thermostable lipases with desired substrate specificity and optimum working conditions continues with two options. First for specific requirement isolate a new enzyme or engineer the existing cloned lipases for desired property. In the past several years, many researchers have employed a variety of molecular tools to improve the catalytic function of the enzymes [20-22]. For instance, a triple mutant of the lipase from Rhizopus arrhizus (RAL) with improved thermostability and a 10°C increased temperature optimum was identified after mutagenesis by epPCR, DNA shuffling and subsequent screening of desired variants [23,24] improved the thermostability of lipase B from Candida antarctica, probably the lipase with the highest number of applications in biocatalysis and lipid modification.

Most of the thermostable lipases have been produced either on the cost of enzyme activity or marginal enhancement in enzyme activity [25]. Therefore, there is a need to evolve existing well characterized lipases with unique modified properties and to study its structure-function relationship. It will provide researchers, a better understanding at molecular level.

In our previous reports we had described about cloning and characterization of LipR1 protein and its evolution for thermostability [16-18]. The evolved proteins LipR2 and LipR3 were thermostable and having single point mutations S311C and R214C [16,17]. Among them LipR3 was more thermostable and LipR2 showed high specific activity. Therefore, an attempt has been made in the present investigation to generate a double mutant harbouring the properties of both the mutants and to examine the cumulative effect of these two mutations on enzymatic properties including the thermostability of the enzyme.

Materials and Methods

Reagents/kits/vectors

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). Gel extraction kit was purchased from RBC (Taiwan). Taq DNA polymerase (1 U/µl) and dNTP mix (25 mM each), were purchased from Fermentas (Germany). QuikChange® XL Site-Directed Mutagenesis Kit was obtained from Stratagene. Substrates (pNP-esters and tributyrin), used for the biochemical assays and screening, were purchased from Sigma Aldrich (USA). All other chemicals used in the study were of good analytical grade, obtained from Merck (Germany).

Site directed mutagenesis and construction of mutagenic library

Site directed mutagenesis was carried out by means of QuikChange® XL site-directed mutagenesis kit, according to the manufacturer's instructions. The PCR reaction was carried out in a Bio-Rad thermal cycler at 95°C for 3 min followed by 18 cycles consisting of 95°C for 50 sec, 60°C for 50 sec and 72°C for 1.25 min and then 7 min at 72°C for final extension. Plasmid of LipR3 was used as template with forward and reverse primers for mutagenesis. Primers were designed to insert the mutation of LipR2 into LipR3.

Forward primer 5' GGCATTGACTGCCATTGGCTT 3'

Reverse primer 5' AAGCCAATGGCAGTCAATGCC3'

The amplified fragments were already in pGEM-T vector so it was used to transform E. coli DH5α competent cells, to obtain a mutagenic library.

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), using an automated AB1 3100 genetic analyzer, that uses fluorescent label dye terminator, based on dideoxy chain termination method. Upon sequence analysis, the mutation S311C was confirmed at the desired position. The open reading frame (ORF) of selected lipase gene was cloned for intracellular expression in pQE-30 UA expression vector (Qiagen, Germany) using primers designed for intracellular cloning by excluding the signal sequence (Forward primer 5'-GGATCCATGGCATCTCGACGC-3' and Normal reverse primers), and expressed in E. coli M15 cells containing pREP4 plasmid as per manufacturer's instructions. The double mutant carrying the mutations of LipR2 and LipR3 was named as LipR4.

Expression and Purification of LipR4

All steps for purification of LipR4 enzyme was carried out at 4°C (unless otherwise stated). LipR1 was also purified simultaneously for comparison. The E. coli M15 cells harbouring the clones in pQE30-UA vector were grown in 200 ml LB media containing 100 µg/ml ampicillin and 30 µg/ml Kanamycin, at 37°C to log phase (A600 0.45-0.5). Thereafter, the cultures were induced by addition of 0.1 mM IPTG and cells were allowed to grow for 3 h at 37°C with shaking. Cells were harvested by centrifugation at 10,000 rpm for 20 min. The cell pellet was suspended in ~ 16 ml of lysis buffer (50 mM Tris-Cl, 0.1 mM Triton-X 100), homogenised and kept for 3 h. The cell suspension was then lysed by sonication (Misonix ultrasonic liquid processor, Model-S 4000) for 10 min (10 sec ON/OFF pulses). The lysate was centrifuged at 10,000 rpm for 20 min to remove cell debris. The supernatant containing soluble fraction of protein in active form was loaded on Ni-NTA column pre-equilibrated with buffer (50 mM Sodium phosphate, 300 mM NaCl, 10 mM imidazole, pH 8.0). The column was washed with two volumes of washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 50 mM imidazole, pH 8.0). The recombinant protein with His-tag at N-terminus was eluted with elution buffer containing 300 mM imidazole in equilibration buffer. The imidazole and other salts were removed by dialysis in 2 L of 10 mM Sodium phosphate buffer kept for 16 h at 4°C, and then it was concentrated in PEG 8000 and stored at 4°C in 50 mM Sodium phosphate buffer (pH 8.0).

Enzyme assay and protein estimation

All the enzymatic assays for determination of residual and relative enzyme activities were carried out according to the modified method of Sigurgisladottir et al. To 0.8 ml of buffer (0.05 M Sodium phosphate buffer,pH 8.0 and 0.1% w/v sodium-deoxicholate),0.1 ml enzyme and 0.1 ml of 0.002 M p-nitrophenyl laurate (dissolved in ethanol) was added. The reaction was carried out in micro-centrifuge tubes at optimum temperatures of the respective enzymes for 10 min, after which 0.25 ml of 0.1 M Na_2CO_3 was added to stop the reaction. The mixture was centrifuged at full speed for 5 min and the activity was determined by measuring absorbance at 420 nm in UV/VIS spectrophotometer (JENWAY 6505 UK). The reaction was measured against an enzyme-free blank to subtract auto-hydrolysis. The molar extinction coefficient for p-nitrophenol in 0.05 M Sodium phosphate buffer (pH 8.0) was 22212 $M^{-1}cm^{-1}$. 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 mole 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.

Biochemical characterization

Polyacrylamide gel electrophoresis

The dialyzed protein was analyzed for purity under denaturing condition on 12% SDS-PAGE gel [26].

Effect of temperature on enzyme activity and stability

Optimum temperature for the LipR4 lipase was determined by assaying the enzyme activity at different temperature (20°C-80°C).

For stability assays, the enzyme was incubated at these temperatures for 30 min, followed by cooling at 4°C for 15 min. The enzyme without incubation was taken as control (100%), and assayed as per standard protocol.

Effect of pH on enzyme activity and stability

Optimum pH for the purified lipase (LipR4) 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. 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 at 50°C.

Thermal inactivation of enzyme

Thermal denaturation profile of the enzyme was studied by pre-incubating the enzymes separately, at 50°C, 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/ the enzyme activity at the 0 time point was taken as control (100%) and the residual lipase activity after incubation was determined. Reaction mix without enzyme served as blank.

Substrate Specificity

Substrates specificity for LipR4 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.

Effect of organic solvents

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. Reaction without enzyme was taken as blank and reaction without organic solvent was taken as control.

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. We have also studied the effect of DEPC (Histidine modifier,1 mM), βME(mM) and esserine (mM) on enzyme activity.

Kinetic Parameter

Enzyme activity as function of substrate concentration (0.01 mM- 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 $K_{cat/Km}$ were also calculated for LipR4, and the result was compared with LipR1.

Biophysical Characterization

Effect of temperature on enzyme conformation (secondary and tertiary) was studied by Circular dichroism (CD) and fluorescence spectroscopic techniques respectively. Circular dichroism is a sensitive probe, which is used to study conformational changes in the secondary structure during the protein unfolding, while tryptophan, an intrinsic fluorophore is quite sensitive to its local environment and provide global conformational changes in the tertiary structure. Proteins solution from both LipR2 and LipR4 were exposed to temperatures ranging from 20 to 90°C. (CD Data for LipR1 and LipR3 was already available (Kumar et al.,). Circular dichroism measurements were made with a JASCO J-715 spectropolarimeter fitted with a Jasco Peltier-type temperature controller (PTC-348WI). Instrument was calibrated with D- 10 camphorsulfonic acid. The temperature of the protein solution was controlled employing cell holder attached to a Neslab's RTE-110 water bath, with an accuracy of ± 0.1°C. Spectra were collected with a scan speed of 20 nm/min and with a response time of one second. Each spectrum was the average of 8 scans. Far-UV CD spectra were taken in the wavelength range of 200-250 nm, at a protein concentration of 15 µM with a 2 mm path length cell. Fluorescence measurements were carried out on a Shimadzu spectrofluorometer (model RF-540) equipped with a data recorder DR-3. The fluorescence spectra were measured at a protein concentration of 10 µM with a 1-cm path length cuvette. To determine intrinsic tryptophan fluorescence, the excitation wavelength was set at 295 nm and emission spectra was recorded in the range of 300-400 nm or at a fixed wavelength of 338 nm with 5 and 10 nm slit width for excitation and emission respectively.

Molecular Modelling

Since these lipases showed high sequence identity (96%) with B. stereothermophilus P1 (BSP) lipase, whose crystal structure is available, it was possible to develop homology models of the proteins with reliability using Swiss Model. The homology models were energy minimized using Swiss-PDB viewer which implements an empirical force field by partial implementation of GROMOS96 in vacuum with default twenty cycles of steepest descent. The models were validated by means of PROCHECK and VERIFY3D available at http://nihserver.mbi.ucla.edu/SAVES/. The qualities of models were also evaluated by means of PROSA. The predicted tertiary structures were analysed in Coot (Crystallographic Object-Oriented Toolkit) and side chains implicated in hydrogen bonds were checked against rotamer libraries (CCP4 Newletter). Hydropathy profiles of the proteins were calculated by a programme (Hydropathy server) available at http://www.tcdb.org/progs/hydro.php.

The nucleotide sequence for lipr4 have been submitted to GeneBank with accession no.

GenBank: KC770104

Results and Discussion

Although there is no any hard and fast rule for thermostability of protein, though there are certain points which tell about structural stability and flexibility of a proteins structure. Among them hydrophobicity and hydrophobic interaction are one of the important parameter. Presence of hydrophobic amino acids inside the core of a protein or on the surface is most often found determining factor for stability and flexibility of a protein. Cystein is an enigmatic aminoacid, as it often shows both types of properties (hydrophobic as well as hydrophilic). Cysteins at the surface are often found to be hydrophilic [10]. However, it is not a fixed rule [16]. Cystein inside the protein behaves as a hydrophobic residue.

Our focus was to see the cumulative effect of two single point mutations. And to study the combined behavior of Cystein residues near the surface. We were able to successfully generate a double mutant (LipR4) by site directed mutagenesis, and studied its biochemical properties in detail.

Cloning of lipr4 Gene for Intracellular Expression of Protein

The plasmid extracted from the lipr4 clone was used for transformation of E. coli M15 ultra-competent cells. The colony PCR of randomly selected clone was carried out to check the presence of the insert (Figure 1). Amplified product of approximately 1.2 kb was seen in 1.2% agarose gel. The DNA was isolated and kept for further use.

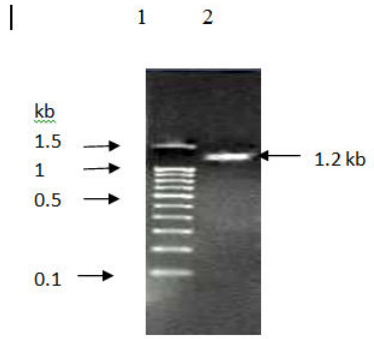

Figure 1: Analysis of Colony PCR product on 1.2% agarose gel. Lane 1- 100 bp DNA ladder, Lane 2- amplified product of approximately 1.2 kb.

Expression and Purification of LipR4 Enzyme

The LipR4 protein was overexpressed at 37°C, using 0.1 mM IPTG. Expression of the protein was checked on 12% SDS-PAGE. The protein was expressed as soluble protein and maximum expression of the protein was obtained after 3 h of induction (Figure 2a). LipR4 protein was purified, as described in methods. LipR1 protein was purified simultaneously along with LipR4 for comparison (Table 1). The LipR4 protein was purified with 52% yield. Purification fold of LipR4 was 66. Specific activity of purified LipR1 and LipR4 enzymes were 8756 and 10261 U/mg respectively. It was interesting to note that the specific activity of LipR4 has decreased drastically when compared to LipR3 (39905 U/mg) and LipR2 (2652914 U/mg) [16].

The protein was purified in soluble form and after dialysis the protein was concentrated to 0.8 mg/ml. The purified protein was checked for homogeneity on SDS-PAGE. The approximate molecular weight of the mutant protein was found to be 44.0 kDa (with His-tag) and the protein migrated at the same position as a single band in 12% SDS-PAGE (Figure 2b).

Purification step	Total protein (mg)	Total Enzyme activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Cell lysate					
LipR1	121 ± 12	14671 ± 98	121	100	1
LipR4	103 ± 12	15970 ± 76	155	100	1
Ni-NTA	Purified				
LipR1	0.8 ± 0.04	7005 ± 78	8756	48	72
LipR4	0.8 ± 0.04	8209 ± 38	10261	52	66

Table 1: Purification table of LipR4 protein.

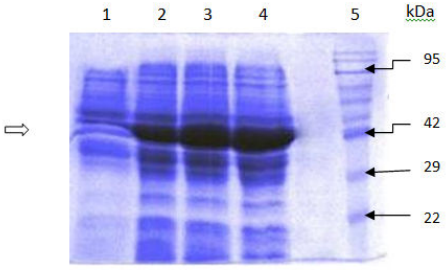

Figure 2a: Expression analysis of LipR4. Lane 1, Uninduced ; Lane 2, culture lysate of LipR4 after 1 h of induction; Lane 3, culture lysate after 2 h of induction, Lane 4, culture lysate after 3 h of induction and lane 5, protein molecular weight marker.

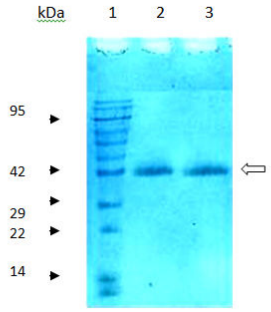

Figure 2b: SDS-PAGE (12%) analysis of LipR4. Lane 1, protein molecular weight marker; Lane 2, purified protein LipR1; Lane 3, purified protein LipR4.

Biochemical Characterization of LipR4 and Comparison with LipR1

Effect of pH on enzyme activity and stability

The LipR4 protein displayed more than 80% enzyme activity at pH 7-9. The maximum activity was found to be at pH 8.0 that was comparable to and LipR1 (8.0) (Figure 3A). The enzyme LipR4 showed little change in pH stability. LipR1 was stable in the pH range of 8.0-9.0, while LipR4 was stable in the pH range of 7.0-10.0 (Figure 3B). The LipR4 protein was more stable at pH 6.0 than LipR1. Stability towards acidic pH can be attributed to addition of Cys residue that has high pKa value and can decrease the pH of enzyme stability.

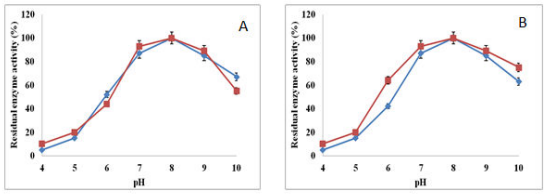

Figure 3: Effect of pH on the activity **(A)** and stability **(B)** of LipR1 **(♦)** and LipR4 **(■)**. All the experiments were performed in triplicates).

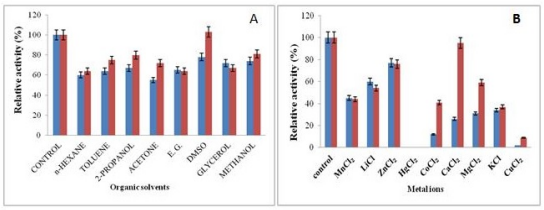

Figure 4: Effect of organic solvents **(A)** and metal ions **(B)** on the activity of LipR4 (Red) and LipR1 (Blue) proteins. (All the experiments were performed in triplicates).

Effect of Additives and Inhibitors

Effect of Organic Solvents

Stability of enzymes in different organic solvents was studied after incubating the enzymes for 1 h (Figure 4A). In presence of select organic solvent (10%, v/v), the residual enzyme activity of LipR4 was relatively higher than the LipR1 protein (Figure 4A). LipR4 enzyme showed little inhibition in DMSO (1%) compared with LipR1 (30%). Both the enzymes showed more than 50% residual enzyme activity with all the solvents tested. Our findings are in correlation with the previous studies where it had been established that there could be a correlation between thermostability and organic solvent tolerance [27].

Most of the metal ions had some inhibitory effect on enzyme activity of LipR4 and LipR1 enzymes. However, LipR4 showed 100% activity with Ca++. LipR4 displayed more than 50% enzyme activity with $MgCl_2$ and ~40% enzyme activity with $CoCl_2$. Both the enzymes displayed more than 70% enzyme activity with $ZnCl_2$ (Figure 4B). The calcium-induced increase on lipase activity (LipR4) could be attributed to the complex action of calcium ions on the released fatty acids, and on enzyme structure stabilization due to the binding of calcium ions to the lipase. It is known that calcium enhances lipase activity.

Effect of Detergents and Inhibitors

Effect of different detergents had been studied on activity of LipR4 enzyme (Table 2). LipR4 showed resistance to selected detergents. It showed 21% inhibition with Tween 40, 22% with Tween 60 and only 11% with Tween 80. It appeared more resistant to Triton-X and SDS than LipR1. LipR2 and LipR3 proteins were also showing resistant to some detergents like Tween 60 and Tween 80. Since the active site contains a serine residue therefore PMSF was used as an inhibitor to test its effect on enzyme activity. Like LipR2 and LipR3, PMSF showed temperature dependent inhibition of enzyme activity with LipR4 (Table 2). LipR4 enzyme activity was not inhibited at 50°C even with 10 mM of PMSF. At 55°C, LipR4 enzyme activity was inhibited by ~20% with 10 mM PMSF. We propose a temperature switch mechanism for inhibition with PMSF. At high temperature the lid become partially open and PMSF enters to active site pocket, thereby inhibiting the enzyme activity. Both, LipR1 and LipR4 showed strong inhibition in the enzyme activity with 1 mM DEPC that showed easy accessibility of histidine residue.

Relative activity (%)				
	LipR1		**LipR4**	
Control	100 ± 2		100 ± 3	
Inhibitors (1 mM)				
DEPC	09 ± 1		14 ± 1	
β ME	98 ± 5		99 ± 3	
Eserine	100 ± 3		99 ± 1	
PMSF*	(1 mM)	(10 mM)	(1 mM)	(10 mM)
At 50°C	89 ± 2	73 ± 4	96 ± 1	98 ± 2
At 55°C	52 ± 5	38 ± 1	100 ± 7	79 ± 6
Detergents (1% V/V)				
Tween 20	51 ± 0		58 ± 1	
Tween 40	68 ± 2		79 ± 2	
Tween 60	27 ± 2		78 ± 6	
Tween 80	39 ± 1		89 ± 3	
Triton-X 100	37 ± 2		76 ± 4	
SDS (1% W/V)	53 ± 4		62 ± 4	
Sod. deoxicholate (1% W/V)	60 ± 5		67 ± 3	

Table 2: Effect of different additives on enzyme activity of LipR4 and LipR1.

Substrate Specificity

The enzymes LipR4 and LipR1 were tested for its activity with different pNP esters. LipR4 showed broad range of substrate specificity with more than 70% enzyme activity with C-14 and ~50% enzyme activity with C-16. LipR1 showed only 30% enzyme activity with C-14 and less than 5% activity with C-16. Both the enzymes displayed maximum enzyme activity with C-12 (Figure 5). LipR2 and LipR3 also showed maximum enzyme activity with C-12. But it was different in other thermostable lipases reported in the literature. While laurate (C-12) was the optimal substrate for this lipase and its variants, B.

stearothermophilus L1 showed maximum activity with p-nitrophenyl-caprylate (C-8). Similarly the lipase from TW1, a thermophillic *Bacillus sp.*, displayed maximum activity with p-nitrophenyl-deconoate (C-10) [28,29].

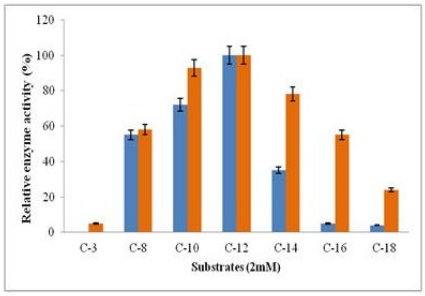

Figure 5: Substrate specificity of LipR4 (Orange) and LipR1 (Blue) proteins, All the experiments were performed in triplicates).

Kinetic Analysis

Effect of substrate concentration on enzyme activity was investigated (Table 3). The enzyme displayed maximum velocity of reaction at 2 mM concentration after which no further increase in velocity of the enzyme reaction was observed. The *Km* (Michaelis-Menten constant) and V_{max} (maximum velocity) values for LipR4 lipase were calculated to 0.71 µM and 607 µmoles/minutes/ml respectively using Lineweaver-Burk plot. The kcat and kcat/Km values for the purified lipase were calculated to be 5472 min-1 and 7707 µM-1 min-1 respectively. There was decrease in the Km value for LipR4 as compared to LipR1. Catalytic efficiency of LipR4 was highly compromised as compared to LipR2 (Table 4) [16]. There was increase in catalytic efficiency of LipR4 as compared to LipR1 (Table 3).

Kinetic Parameters	LipR1	LipR4
Km (mM)	0.78 ± 0.02	0.71 ± 0.03
Vmax (mmoles min-1 ml-1)	502 ± 7	607 ± 3
kcat (min-1)	977 ± 11	5472 ± 17
kcat/Km (mM-1 min-1)	1253 ± 13	7707 ± 23

Table 3: Kinetic parameters of LipR4 and LipR.

We were expecting retaining of catalytic efficiency of LipR2 in LipR4, but it was not the case. In LipR2, authors had proposed a channel like region leading to active site that could be reason for its high specific activity [16]. In LipR4, we observed loss of channel like region. That may be the cause for low specific activity of LipR4 than LipR2 (Table 4).

Biochemical parameters	LipR1	LipR2	LipR3	LipR4
Optimum pH	8	7	8	8
pH stability	8.0-10.0	7.0-9.0	8.0-10.0	7.0-11.0
Optimum Temp	50°C	45°C	45°C	50°C
Substrate specificity	C-12	C-12	C-12	C-12
Specific activity (U/mg)	8756	3E+06	39905	10261
Half life at 50°C	2 h	12 h	18 h	16 h
Half life at 60°C	5 min	4.5 h	7.5 h	4 h
Half life at 65°C	< 5 min	27 min	32 min	10 min
References	-16	-16	-17	Present study

Table 4: Comparison of biochemical properties of LipR4 with other lipases.

Effect of Temperature on Enzyme Activity and Stability

The enzyme LipR4 showed temperature optima of 50°C (Figure 6A) similar to that of LipR1. Thermostability studies of LipR4 was carried out at different temperatures as mentioned in methods and compared with LipR1 as well as LipR2 and LipR3. LipR4 was quite stable in the temperature range of 50°C-60°C. The half-life at 50°C of LipR4 was found to be ~16 h, much greater than LipR1 (2 h) and LipR2 (12 h) (Table 4) but was less than LipR3 (18 h). At 60°C, LipR4 showed half life of 4 h, much greater than LipR1 protein (5 min) (Figure 6B and 6C) but, was comparable to LipR2 (4.5 h), again it was much less than LipR3 (7.5 h). At 65°C, LipR4 displayed half life of 10 minutes, whereas LipR3 displayed half life of 32 min., LipR1 was not comparable as it lost activity instantaneously (Figure 6D). It was interesting to observe that the stability/half life of LipR4 protein at 60°C had been shifted towards LipR2. Although, LipR4 is more thermostable than LipR1 though, it has less thermostability than LipR2 and LipR3. LipR3 itself was highly thermostable but after incorporation of mutation of LipR2, its thermostability had shifted drastically.

Circular dichroism (CD) and fluorescence studies

To study the changes at secondary and tertiary structure level, we compared the CD data of LipR4 with other variants. Far-UV CD spectra for LipR4 displays strong negative bands in the region of 200-250 nm. The protein (LipR4) showed minimal loss in secondary structure on increasing temperature from 20°C to 40°C (Figure 7D). However, loss in secondary structure was observed at 50 and 60°C. Thus LipR4 structure was more robust as compared to LipR1 (Figure 7A and 7B), but was less stable than LipR2 and LipR3 (Figure 7C) at 60°C. Investigation of structural changes using intrinsic tryptophan fluorescence as a function of temperature (Figure 7E and 7F), it was observed that LipR4 displayed gradual decrease in intensity. In case of LipR1 [17], the emission maxima drastically decreased after 30°C indicating significant unfolding, while in case of LipR4 little change in emission maxima was observed even up to 50°C. These studies clearly indicated that LipR4 is more thermostable as compared to LipR1. But it was less thermostable than LipR2 and LipR3.

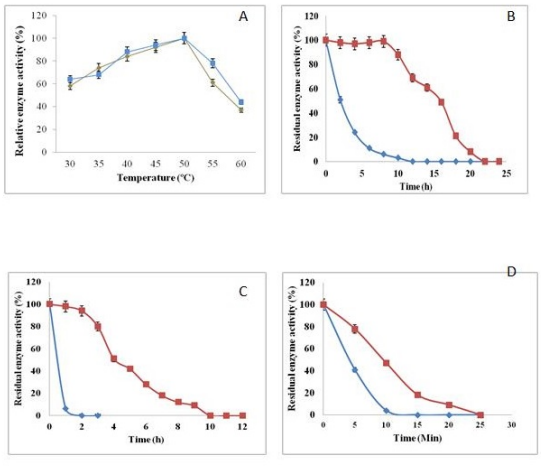

Figure 6: Effect of temperature on enzyme activity (A) and stability of LipR1 (♦) and LipR4 (■) enzymes at 50°C **(B)**, 55°C **(C)** and 60°C **(D)**. All the experiments were performed in triplicates).

Structure analysis by homology model and investigation of cumulative effect of single point mutations on thermostability of Protein

Structure analysis of the variant (LipR4) showed that, the substitution S311C is located at the junction of a 310 helix (311-314) and loop connecting α11 (Figure 8A and 8B). The effect of mutation S311C has been previously reported by Kumar et al., (2013a), where Cys had been found to involve in generation of a hydrophobic core thereby enhancing the enzyme activity and providing rigidity to the protein. But in LipR4, the side chain of Cys 311 (Figure 8C and 8D) has moved towards the exposed surface and seems to be involved in destabilization of hydrophobic core. The other mutation R214C is located in the loop (191-220) which connects α6 (Lid helix) and α7. Kumar et al., (2013b) have shown that in LipR1, Arg 214, is unable to pack with the compact hydrophobic core and thus interacts with bulk solvent, thus acts as a destabilizing residue. Substitutions of Arg with Cys (R214C) lead to packing of Cys favourably with this hydrophobic compact core and thus providing stability to protein structure by relieving the entropic strain due to Arg (A comparative analysis of LipR4 with all the variants had been shown in Figure 8). Individually these substitutions had provided stability to proteins by either forming H-bonding with neighbouring amino acids (R214C) or by enhancing hydrophobicity of protein structure (S311C). Surprisingly, when these mutations were introduced together in a gene (lipr4), they didn't demonstrated additive effect on thermostability.

In homology model of LipR4, we also observed that side chains of Cys311 is fully exposed to bulk solvent that is energetically unfavourable (Figure 8C) and -SH group of Cystein is exposed towards surface that is easily available for oxidation. And this may be a big reason for less thermostability of LipR4 than lipR3. In case of LipR2, the side chains of Cys were protruding towards inner core and was making channel for substrate accessibility [16]. But in this case, Cys311 was unable to make such channel; this may be a good reason for its low enzymatic activity than LipR2. The side chains of Cys 214 have not changed in LipR4 as compared to LipR3 and LipR1 (Figure 8D and 8E).

We were successful in enhancing thermostability at 50°C, but above 60°C we couldn't observe an additive effect. (Data is also supported by our CD and fluorescence studies, where, we observed that LipR4 is maintaining its helical properties till 60°C (Figure 7). In a similar attempt to enhance thermostability we were able to get additive effect of single point mutations [18] on thermostability. In this case, although the reason for decrease in thermostability of LipR4 as compared to LipR3 is not well understood, though, some assumption has been made. One of the prominent reasons may be due to increased presence of thermolabile amino acids (Cys) at the surface (Figure 7A and 7B). There are reports where it was shown that the thermolabile amino acids like Cys and Asn at the surface readily undergo oxidation and amination, respectively, and therefore decrease the protein thermal stability [10,13,15]. In this case two Cys residues along with other thermolabile amino acids like Asn304 (on the surface) might be responsible for the destabilization of LipR4.

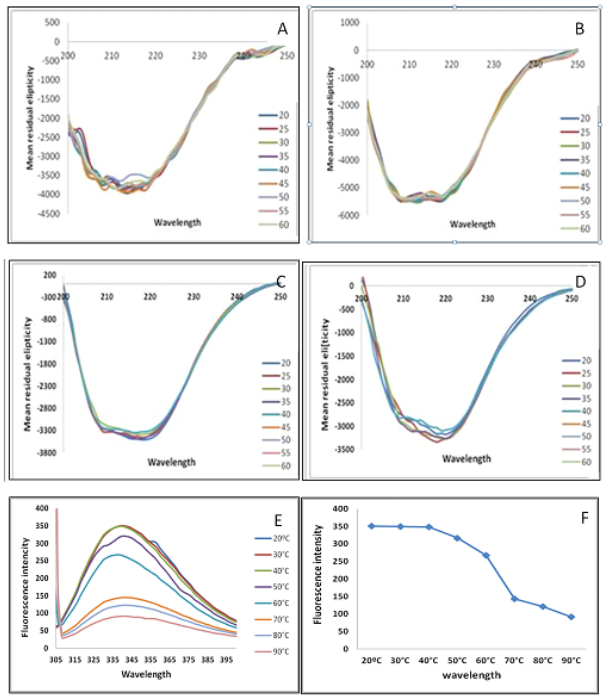

Figure 7: Far UV-CD of lipases recorded in the 250-200 nm range from 20 to 60°C for LipR1 **(A)**, LipR3 **(B)**, LipR2 **(C)** and LipR4 **(D)**. Effect of temperature, on intrinsic tryptophan fluorescence of LipR4 (E) in the range of 300 to 400 nm. Fluorescence spectra of LipR4, recorded at a fixed wavelength of 338 nm **(F)**.

There are several reports demonstrating an improvement of a property independently of another one, e.g. thermo stabilization without sacrificing catalytic activity [16,30-32]. These reports provide case studies where there was successful development of promising mutants of their target enzymes without losing other properties. Yet, these approaches are stochastic approaches, which are highly dependent on the characteristic and potential of target enzymes. Methods applicable to a wide variety of enzymes are still not available.

There has been some promotion of the idea that accumulation of advantageous mutations based on the near-additivity is an approach suitable for making a significant improvement in desired protein property [33]. Some reports theoretically describe the near-additively feature of advantageous effects of multiple mutations in local fitness landscapes [34,35]. It has been predicted quite early that accumulation of mutations, which exclusively contribute to a certain property, would be one of the approaches for improving a specific property [36].

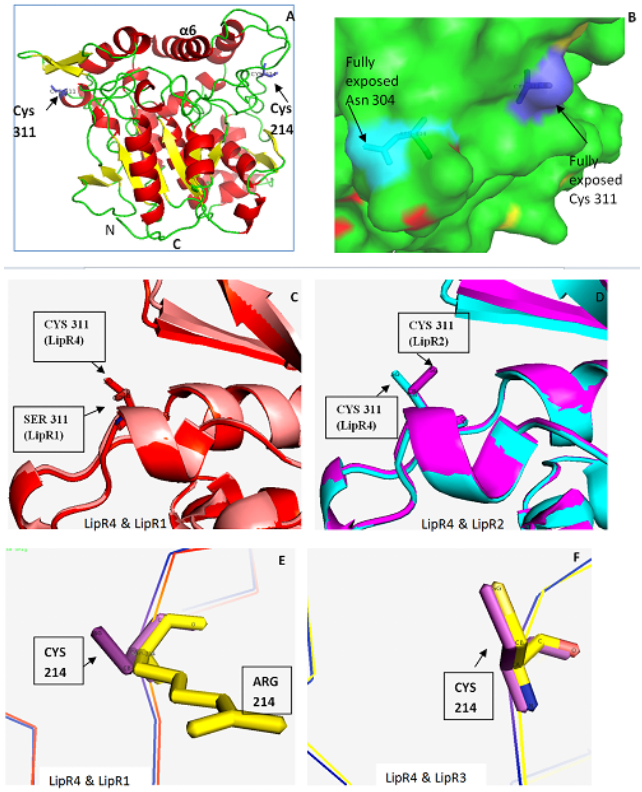

Figure 8: Structural analysis of mutations in LipR4 by homology modelling. Cartoon diagram showing LipR4 model with substituted amino acids (R214C) and (S311C) (A). A snapshot of surface representation with 20% transparency, illustrating the position of surface exposed Asn 304 and Cys 311 (B). A section of cartoon representation, showing comparison of side chains of Cys 311 of LipR4 (Red) and Ser 311 of LipR1 (C). A section showing comparison of side chains of Cys 311 in LipR4 (Cyan) and LipR2 (Magenta) (D). A section of Ribbon representation, showing side chains of Cys214 and Arg 214 in LipR4 (Magenta) and LipR1 (Yellow) respectively (E). Ribbon representation showing Side chains of Cys 214 in LipR3 (Yellow) and LipR4 (Magenta) (F).

Conclusion

Our study clearly suggest that additive effects of multiple mutations in order to enhance a desired property is still a stochastic process and will differ from case to case as well as depend upon the position, nature, context of mutation which might influence protein thermostability in a yet unknown manner.

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