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Characterization and molecular modelling of an engineered organic solvent tolerant, thermostable lipase with enhanced enzyme activity

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ABSTRACT

To evolve a variant with improved enzymatic properties i.e. thermostability, catalytic efficiency, by error prone mutagenesis, *lipR1* was used as template. Using two tier screening protocol, a highly thermostable lipase variant (LipR3) with a single amino acid change (R214C) was identified. The LipR3 lipase demonstrated 9 folds enhanced catalytic efficiency in comparison to LipR1 enzyme. The variant demonstrated 90 folds higher thermostability at 60 °C, when compared with the parent enzyme. In addition it displayed appreciable tolerance to organic solvents. The variant gene was cloned in pQE30-UA and the protein was expressed in *E. coli*. The protein product of approximately 44.0 kDa was purified by affinity chromatography and characterized in detail. Temperature dependent circular dichroism and steady state fluorescence studies of LipR3 and LipR1 lipase supported the observation that LipR3 is more thermostable than LipR1. Structural analysis of the homology model of LipR3 showed that the Arg 214 was substituted with Cys at a large surface loop which connects lid helix (α6) to another helix (α7). The hydrophobic residues of the loop (Val 197, Tyr 204 and Phe 206) formed a compact hydrophobic region with residues from lid helix. It is suggested that replacement of Arg with Cys leads to packing of Cys favourably with hydrophobic core and provide structural stability to LipR3.

1. Introduction

The structural stability of proteins involves a delicate balance between the favourable internal interaction among residues of the protein and the protein–solvent interaction. Many proteins optimize their structural stability in an aqueous environment. When submerged in organic solvents or placed at immiscible interfaces, their native structures are likely to be weakened and their activities diminished [1]. But for a few proteins such as lipases (triacylglycerol acylhydrolases, EC 3.1.1.3), that interact directly with an organic substrate at the interface [2], the scenario is different. The enzymes remain active in organic phase and catalyze the reactions (esterification and transesterification) efficiently.

Adequate thermostability, catalytic efficiency and stability of enzymes in organic solvents are the prerequisite for industrial applications in biotechnology [3,4]. Often some of these properties are compromised in naturally occurring enzymes [5,6]. Rational protein engineering using site-specific mutagenesis or directed evolution is routinely being used for improvement of enzyme characteristic [7–11]. Several enzymes have been reported to be evolved to demonstrate enhancement in thermostability [12–14]. In most of the cases gain in thermostability was on the cost of enzyme activity [15,16]. There are very few reports showing simultaneous enhancement in more than one property [14,17,18]. Several methods such as chemical modification, protein engineering, etc. have been applied to improve the stability of lipases in organic solvents, though with limited success [19].

Among industrial biocatalysts lipases are one of the most commonly used enzymes. It is a valuable biocatalyst in food, pharmaceutical, detergent, and chemical industries [4]. Lipase successfully catalyzes various reactions which include interesterification, acidolysis, esterification, alcoholysis and aminolysis in addition to its hydrolytic activity on triglycerides. The performance of lipase in the presence of various solvents is related to the efficiency of synthetic and hydrolytic reactions. Esterification and transesterifications are carried out in low-water-content media and using non-polar solvents. Several thermostable lipases have been reported with high optimum temperature and reasonably good stability in organic solvents [6,20,21]. Several of these are accompanied by low specific activity of enzyme [22]. On the other hand very high optimum temperature of enzyme is not suitable for industrial application due to the high temperature for reaction resulting in enhanced cost of production.

In this regard, considerable attention has been paid in development of thermostable lipases with simultaneous improvement of other properties (organic solvent tolerance and enzyme

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activity). We have already cloned a lipase gene from metagenomic source. The enzyme demonstrated optimum activity at 50 °C and pH 8.0. The enzyme was stable for 5 min at 60 °C, for 15 min in 10% organic solvent and demonstrated moderate enzyme activity. It is in this perspective; we report generation of an improved lipase by error prone mutagenesis. An attempt has been made to explain the change in function on the basis of structure modelling. The importance of the study lies in simultaneous improvement of thermostability, organic solvent tolerance and kinetic efficiency with only single point mutation.

2. Materials and methods

2.1. Reagents/kits/vectors

pGEM-T easy vector and pQE30-UA vectors were purchased from the Promega (USA) and Quiagen (Germany) respectively. 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). GeneMorph® II Random 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).

2.2. Error prone PCR and construction of mutagenic library

Error prone PCR was carried out, using GeneMorph® II Random Mutagenesis kit, according to the manufacturer's instructions. A mutation condition that should generate an error frequency of approximately one to two mutations per 1000 bases was used. The PCR 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 s, 60 °C for 50 s and 72 °C for 1.25 min and then 7 min at 72 °C for final extension. Plasmid of wild type (LipR1) was used as template with normal forward (5′-ATGATGAAAGGCTGCAGGGTG-3′) and reverse primers (5′-TTAAGGCCGCAAACTCGCCGTTGCCC-3′). The amplified fragments were already in pGEM-T vector so it was transformed in *E. coli* DH5α cells, to obtain a mutagenic library.

2.3. Screening of the mutagenic library

Mutagenic library was screened for thermostable mutant lipase using two tier screening method. First the mutants were screened on plates then it was confirmed by tube assay. In plate assay method, cells were grown at 37 °C over night on LB amp-kan plates followed by incubation at 60 °C for 1 h. The control plates were kept at 37 °C. Then it was overlaid with 0.5% tributyrin emulsion (0.5% tributyrin, 0.4% agar in water) and kept at 37 °C for 8 h to observe the zone of hydrolysis. The colonies showing larger zone of clearance were subjected to second round of screening by tube assay method. The cells were grown in 5 ml culture tube at 37 °C overnight then it was pelleted down at 12,000 rpm for 10 min. The supernatant was subjected to incubation at 60 °C for 10, 20 and 30 min followed by cooling on ice for 15 min before carrying out enzyme assay by standard assay procedure (discussed in Section 2.6). The culture supernatant without incubation was taken as control.

2.4. Nucleotide sequencing and analysis

To confirm the position of mutation, the plasmid from the selected mutant clone was sequenced using universal M13 forward and reverse primer. The nucleotide sequencing was done from both sides 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 open reading frame (ORF) of selected lipase gene was cloned intracellularly in pQE-30UA expression vector (Qiagen, Germany) using primers designed for intracellular cloning (forward primer 5′-GGATCCATGGCATCTCGACGC-3′ and normal reverse primers). The vector containing insert was transformed in *E. coli* M15 cells containing pREP4 plasmid as per manufacturer's instructions.

2.5. Expression and purification of LipR1 and LipR3

All steps for purification of LipR1 and LipR3 enzymes were carried out at 4 °C (unless otherwise stated). The *E. coli* M15 cells harbouring the clones in pQE-30UA vector were grown in 200 ml LB media containing 100 μg/ml ampicillin and 30 μg/ml kanamycin, at 37 °C to late log phase. Thereafter, the cultures were induced by addition of 0.1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) 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–HCl, 0.1 mM Triton X-100), homogenized and kept for 3 h. The cell suspension was then lysed by sonication (Misonix ultrasonic liquid processor, Model-S 4000) for 10 min (10 s 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 buffer pH 8.0, 300 mM NaCl and 10 mM imidazole). The column was washed with two volumes of washing buffer (50 mM sodium phosphate buffer pH 8.0, 300 mM NaCl and 50 mM imidazole). 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).

2.6. Enzyme assay

All the enzymatic assays for determination of residual and relative enzyme activities were carried out according to the following method. To 0.8 ml of buffer (0.05 M sodium phosphate buffer (pH 8.0) + 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 microcentrifuge 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 22,212 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 μmole 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.

2.7. Biochemical characterization

2.7.1. Polyacrylamide gel electrophoresis

The dialyzed proteins were analyzed for purity under denaturing condition on 12% SDS-PAGE (sodium dodecyl sulphate