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Point mutation Gln121-Arg increased temperature optima of *Bacillus* lipase (1.4 subfamily) by fifteen degrees

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ABSTRACT

Small molecular weight *Bacillus* lipases are industrially attractive because of its alkaline optimum pH, broad substrate specificity and production in high yield by overexpression both in *Escherichia coli* and *Bacillus subtilis*. Its major limitation of being mesophilic in nature is constantly targeted by laboratory evolution studies. Herein metagenomically isolated *Bacillus* LipJ was randomly evolved by error prone PCR and library of variants were screened for enhanced thermostability. Point mutant Gln121Arg was extensively characterized and it showed dramatic shift of Temp. opt to 50 °C compared to 37 °C for parent enzyme. Thermostability studies at 45 °C and 50 °C determined six fold increase in half life for point variant Gln121Arg compared to LipJ. Circular dichroism (CD) and tryptophan fluorescence study established enhanced thermostability of Gln121Arg. Specific activity of point variant Gln121Arg was comparable to wild type with increased substrate affinity (Km reduced). Reduced kcat for variant Gln121Arg infer that kinetic and catalytic efficiency of mutant was compromised. Structural implications by homolog modelling predicted Gln121 to be placed within longest loop of the structure at surface. Localization of loop due to additional polar interactions by Arg121 to protein core defines molecular basis of enhanced thermostability of random point variant Gln121Arg.

1. Introduction

Small proteins unfold completely, reversibly and better permit quantification of mutational effects in terms of thermodynamics as ΔG of folding. Therefore lowest molecular weight lipase from *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus licheniformis* subgrouped in subfamily 1.4 of true lipase [1] are good target for laboratory evolution for enhanced thermostability and structure function analysis [2–6]. The properties of extreme alkaline tolerance and overexpression in *Escherichia coli* make *Bacillus* lipase are industrially desirable. But their mesophilic nature with significant decrease in activity and stability above 45 °C, limits its industrial applications. Two different strategies are effectively used to genetically modify

Bacillus lipases to improve its activity and stability at high temperature 1. Directed evolution [2,5,6] 2. Rational designing [3,4,7]. Directed evolution is mimicry of natural evolution at lab bench, in which variant with improved property is selected through pool of genetically diverse variants under selective conditions. Error prone PCR is the most common directed evolution technique to introduce random mutations.

Protein stability is expressed as energetic of unfolding reaction from native state to fully unfolded state. Low molecular weight *Bacillus* lipases unfold completely and reversibly because disulfide bridges are lacking due to absence of Cys residue. Therefore protein stability can be better quantified for lipase of 1.4 subfamily. Metagenomically isolated *lip* gene (accession number FJ54454.1) cloned in pGEM-T vector was obtained from lab. Our lab is actively engaged in laboratory evolution of

Bacillus lipases for last decade. BLAST analysis determined *lip* gene to share 99% nucleotide identity with secreted esterase of *Bacillus* sp. BP6. *Bacillus* lipase LipJ (ACL78785.1) encoded by *lip* gene was used in present study. Lipase LipJ is of 210 amino acids with 28 residues signal peptide at N-terminal region. Its molecular weight is 19.6 kDa with active site constituents Ser77, Asp133, and His156 (position mentioned is after cleavage of signal peptide). Laboratory evolution of Bacillus lipases is reported in many studies [2–6]. Our laboratory has been actively engaged in the structural

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and functional basis of cold adaptation of *Bacillus* lipase LipJ [37,38]. In present study mutants were generated to screen and select for point thermostable mutant. Mutations were randomly introduced in the gene *lip* by error prone PCR using mutazyme polymerase. Genetically diverse library of

Bacillus LipJ variants was obtained and best suited variant at denaturing temperature 50 °C was selected. Point mutant was preferred among finally selected and sequenced thermostable variants. The mutant was carried forward for extensive biochemical and biophysical characterization. To understand the enhanced thermostability of the mutant, we employed homology modelling to provide a structural basis for the observed effect.

2. Material and method

2.1. Chemicals/biochemicals

All the chemicals and reagents used for the study were of analytical grade and purchased from MERCK (Germany), HiMEDIA (India). T4 DNA ligase, Taq polymerase was purchased from New England biolabs (MA, USA). Ampicillin, kanamycin, IPTG and X-gal were purchased from Hi Media (India). Ni-NTA resin was purchased from Qiagen (Germany). pNP esters, tributyrin substrates were purchased from Sigma Aldrich (USA). Mutazyme I DNA polymerase (Genmorph mutagenesis kit, Agilent technologies, CA) was used to introduce random mutation by error prone PCR.

2.2. Random mutagenesis by error prone PCR

Random mutations were introduced with in *lip* gene using mutant Taq DNA polymerase i.e. mutazyme I DNA polymerase from Genmorph mutagenesis kit as per manufacturer's instructions.

Mutation frequency depends upon the initial amount of target DNA and number of PCR cycles. In order to mutagenize 1.0 kb target gene at low mutation frequency, 500 ng of template DNA was recommended (as per manufacturer's instructions). For 1.0 kb target DNA inserted in 3.0 kb plasmid

DNA (total construct of 4.0 kb), 2 μg of plasmid construct should be amplified for 20–25 cycles to obtain lowest mutation frequency of 0–4.5 mutation/kb. The PCR was carried out in Bio-RAD thermal cycler at 95 °C for 3 min followed by 20 cycles consisting of 95 °C for 1 min, 57 °C for 1 min, 72 °C for 1 min and then 10 min at 72 °C for final extension. Wild type *lip* gene was used as template with normal forward and reverse primers mentioned in table. Amplified library of variants was cloned in pGEM-T Easy vector.

2.3. Screening of library of variants and selection of thermostable variant

Library of error prone variants were screened through three tier screening assay (i) LBT plate assay, (ii) 96 deep well culture plate assay (iii) Liquid eppendorf tube assay. Library of mutants were spread on LBT—amp plate and incubated overnight at 37 °C followed by incubation at 50 °C for 1 h. Control plates were kept at 37 °C. After that, plates were overloaded with 0.5% tributyrin emulsion (0.5% tributyrin, 0.4 agar in water). Colonies showing larger zone of clearance were screened and subjected to second round. For 96 well plate assays, selected colonies were simultaneously inoculated in each well of 96 well culture plates containing LB-amp media and incubated at 37 °C for 24 h. Supernatant was isolated from each clone and was exposed to 50 °C for 10 min followed by cooling at 4 °C and then assayed with pNP-laurate as substrate. Colour produced by enzymatic reaction was monitored on ELISA reader at 420 nm. For each selective step, variants with high relative activity at 50 °C compared to WT were selected and confirmed in subsequent screening steps. Mutagenized clones with improved stability at high temperature were selected and sent for sequencing by commercial available sequence service.

2.4. Intracellular expression and purification

For intracellular expression, Fwd primer complimentary to region downstream signal sequence was designed. Restriction sites compatible to polycloning site of pQE30 were inserted upstream the fwd. Primer with BamHI restriction site and rev. Primer with HindIII restriction site. Amplified mature *lip* gene (without signal sequence) was amplified, digested and ligated to pQE30 vector. Recombinant pQE30 was checked for orientation of gene by colony PCR using T5 promoter primer. pQE30 + mature *lip* was transformed in *E. coli* M15 cells. *E. coli* cells expressing lipase were grown overnite in conical flask containing 100 μg/ml ampicillin and 30 μg/ml kanamycin. Cells were induced with 0.1 mM IPTG overnight at 30 °C. Cells were pelleted and suspended in 16 ml lysis buffer, homogenized and kept for 3 h. The cell suspension was lysed by sonication (Misonix ultrasound liquid processor, Model-S4000). Supernatant containing His-tagged recombinant lipase and its variants were purified to homogeneity by one step purification using immobilized Ni ion affinity chromatography (Bio-RAD). Purified protein was analyzed for purity by SDS-PAGE. Proteins were visualized by Coomassie brilliant blue R250.

2.5. Biochemical characterization of wild type LipJ and point variant Gln121Arg

All the enzymatic assays for determination of residual and relative enzyme activities were carried out according to the method of Kumar et al. [8]. 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 l 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.

2.5.1. Effect of temperature on enzyme activity

Enzymatic activity of lipase was studied at different temperatures (20–60 °C). Enzyme was suitably diluted for enzyme activity. Thermostability assay was carried out by pre incubating enzyme for different time intervals at 45 °C and 50 °C, followed by cooling on ice for 15 min. Then enzyme assay was done at its optimum conditions. Enzyme without incubation was taken as control (100%).

2.5.2. Effect of pH on enzyme activity

Lipase activity was assayed using different buffers of pH (5–10) for variant and parent enzyme. Enzyme assay was carried out according to standard assay conditions.

2.5.3. Substrate specificity

Substrate specificity pattern of wild type and mutant was carried out using pNP esters of chain length C3, C, C8, C10, C12, C14 and C16. The assay was performed according to standard assay method at optimum temperature and pH.

2.6. Kinetic study

Lineweaver Burk plots were drawn for wild type LipJ and variant Gln121Arg. Kinetic parameters (K_m, k_{cat}, V_{max}) were calculated from activity vs substrate concentration curve (pNP laurate 0.01–5 mM). Enzyme activity assay was performed at optimum temperature and pH of enzyme.