Gene 491 (2012) 264–271

Contents lists available at SciVerse ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Short Communication

Engineering of a metagenome derived lipase toward thermal tolerance: Effect of asparagine to lysine mutation on the protein surface

Pushpender Kumar Sharma [a,b], Rajender Kumar [c], Rakesh Kumar [a], Owais Mohammad [d], Ranvir Singh [e], Jagdeep Kaur [a,*]

[a] Department of Biotechnology, Sector 14, Panjab University, Chandigarh 160014, India
[b] Indian Institute of Science Education and Research (IISER) Mohali, S.A.S. Nagar, Sector 81, Mohali-140306, Punjab, India
[c] Computer Centre, National Institute of Pharmaceutical Education and Research (NIPER), Sector-67, S.A.S. Nagar 160062, Punjab, India
[d] Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh 202002, India
[e] National Centre for Human Genome Studies and Research (NCHGSR) Panjab University Chandigarh, 160014, India

ARTICLE INFO

Article history:
Accepted 24 September 2011
Available online 6 October 2011

Received by A.J. van Wijnen

Keywords:
Lipase
Directed evolution
Catalytic efficiency
Far-UV CD
Mutation
Fluorescence

ABSTRACT

A highly thermostable mutant lipase was generated and characterized. Mutant enzyme demonstrated 144 fold enhanced thermostability over the wild type enzyme at 60 °C. Interestingly, the overall catalytic efficiency (k_{cat}/K_m) of mutant was also enhanced (~20 folds). Circular dichroism spectroscopy, studied as function of temperature, demonstrated that the mutant lipase retained its secondary structure up to 70–80 °C, whereas wild type protein structure was completely distorted above 35 °C. Additionally, the intrinsic tryptophan fluorescence (a probe for the tertiary structure) also displayed difference in the conformation of two enzymes during temperature dependent unfolding. Furthermore, mutation N355K resulted in extensive H-bonding (Lys355 HZ1 – OE2 Glu284) with a distance 2.44 Å. In contrast to this, Wt enzyme has not shown such H-bonding interaction.

1. Introduction

Lipases (Triacylglycerol hydrolases) belong to a group of enzymes, which catalyze the synthesis and hydrolysis of long chain fatty acids (Rubin and Dennis, 1997). These are among the most versatile studied enzymes, and are used in a number of applications in various industries, e.g. pharmaceutical, dairy, detergent, cosmetic, oleochemical, fat-processing, leather, textile, cosmetic, and paper industries (Gerhartz, 1990). All lipases consist of a compact minimal α/β hydrolase fold. The active site of the α/β hydrolase fold enzymes contains, a nucleophilic residue (serine), a catalytic acid residue (aspartate/glutamate), and a histidine residue, always in this order in the amino acid sequence (Ollis et al., 1992). All natural occurring lipase enzymes do not meet industrial requirement, e.g. improved activity and enhanced thermal stability. Therefore, there is always continuous need to evolve the enzyme with better activity and thermostability. Alteration of the pre-existing genes in this context can play an important role in evolving enzymes with desired function. Though, there might be multiple ways to manipulate and alter the catalytic function of an enzyme, the approach that minimizes the efforts is preferred (Bergquist et al., 2005; Camps et al., 2003; Hamamatsu et al., 2005; Ostermeier et al., 1999; Peisajovich and Tawfik, 2007; Peisajovich et al., 2006; Sen et al., 2007; Volles and Lansbury, 2005). In past several years directed evolution of biocatalysts has emerged as method of choice for improvement of structural and functional properties of the enzymes (Boersma et al., 2007; Chatterjee and Yuan, 2006; Johannes and Zhao, 2006). Diverse class of industrially important enzymes have been modified for enhanced thermostability, by directed evolution that include e.g., esterase's, lipases, amylases and xylanases (Andrews et al., 2004; Machius et al., 2003; Spiller et al., 1999; Zhang et al., 2003). Factors known to enhance the proteins thermal stability include, the hydrophobicity profile, the number of hydrogen bonds, the amino acid composition, their distribution and interactions in the protein (Vieille and Zeikus, 2001). Furthermore, comparison of the protein from thermophiles and mesophiles can also play an important role in the predictions of stabilizing mutation (Cambillau and Claverie, 2000; Vogt et al., 1997). However, no single traffic rule for correlation of thermostability with particular amino acids can be generalized (Nawani and Kaur, 2007). It was further stated that the

Abbreviations: CD, Circular dichroism; IPTG, Isopropyl-beta-thio-galactopyranoside; PMSF, Phenylmethylsulphonyl fluoride; DEPC, Diethyl pyrocarbonate; pNP, Para-nitrophenyl; SDS, Sodium dodecyl sulfate; PDB, Protein database; NCBI, National Centre for Biotechnology Information; BLAST, Basic Local Alignment Search Tools; SAVES, Structural Analysis and Verification Server; ORF, Open reading frame; OD, Optical density; 3D, Three dimensional; Asn (N), Asparagine; Lys (K), Lysine.

* Corresponding author at: Dept. of Biotechnology, Sector 14, Panjab University, Chandigarh 160014, India. Tel.: +91 172 2534086; fax: +91 172 2541409.
E-mail address: jagsekhon@yahoo.com (J. Kaur).

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doi:10.1016/j.gene.2011.09.028

energy associated with the stability of the protein is small, as the difference between the native and denatured state of protein is only a matter of few weak interactions (Jaenicke, 1996, 2000; Jaenicke and Bohm, 1998).

In the present investigation, we have modified a Wt (wild type) lipase gene (JkP01) by error prone PCR, which was initially cloned from metagenomic DNA, extracted and purified from hot spring (Sharma et al., 2007). Our results on Wt lipase demonstrated that enzyme was stable up to 50 °C, and its activity declined sharply at 60 °C ($t^{1/2}$ ~5 min) (Sharma et al., 2011). Attempts were therefore made to generate a thermostable mutant lipase using Wt gene, by error prone PCR. A mutagenic library was constructed and screened, functional screening of the library resulted in a mutant lipase (lip M1), with mutation N355K in the mature polypeptide. The lip M1 demonstrated 144 folds enhanced thermostability over the Wt enzyme, when assayed at 60 °C. Both enzymes (lip M1 and Wt) were characterized in detail using biochemical, biophysical and computational approaches.

2. Materials and methods

2.1. Molecular manipulations, protein expression and purification

2.1.1. Reagents/kits/plasmids

pGEM-T easy vector used for cloning purpose was purchased from the Promega (USA). pQE30-UA plasmid, used as expression vector was purchased from the Quiagen (Germany). Gel extraction kit was purchased from MOBIO (USA). *Taq* DNA polymerase (5U/μl) and dATP, dGTP, dCTP, dTTP (25 mM each), each were purchased from the Fermentas (Germany). 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).

2.1.2. Oligonucleotides

Oligonucleotides used for PCR amplification of lipase gene with terminal signals were as follows; 5′-TGATGAARGGNTGYAGRGTNCC-3′ (forward primer) and 5′-TTANGGNCGNA (A/G) N(C/G) (T/A) NGCNA (G/A) (T/C) TGNCC-3′ (reverse primer). Primer sequence used to clone the gene without terminal signal was 5′-GGATCCATGG-CATCTCGACGC-3′. All the primers were used in final concentration of 0.4 μM/25 μl reaction for PCR amplification.

2.1.3. Error prone PCR and construction of mutagenic library

Error prone PCR was performed in presence of 7 mM $MgCl_2$ and varying concentration of four dNTPs, as described by Leung et al. (1989). The PCR (gradient) reaction was carried out in a Bio-Rad thermal cycler, at 94 °C for 4 min, followed by 30 cycles, at 94 °C for 1 min, 55/59.5 °C for 50 s and 72 °C for 2 min with a final extension of 10 min at 72 °C. PCR amplified product was cloned in pGEM-T easy vector followed by its transformation in *E. coli* DH5α cells, to obtain a mutagenic library.

2.1.4. Enzyme assay

Enzyme assay was performed according to Sigurgisladottir et al. (1993). To 0.8 ml of 0.05 M phosphate buffer (pH 8.0), 0.1 ml enzyme and 0.1 ml of 0.002 M p-nitrophenyl laurate was added, followed by mixing and incubation at 50 °C for 10 min. Reaction was stopped by adding 0.1 M Na_2CO_3 (0.25 ml). Reaction mixture was centrifuged and supernatant was used to determine the enzyme activity. Enzyme activity was measured at 420 nm in UV/Vis spectrophotometer (JENWAY 6505, UK). Enzyme activity was measured at 420 nm in UV/Vis spectrophotometer (JENWAY 6505, UK). One unit of enzyme activity is defined as the amount of enzyme, which liberates 1 μ mole of p-nitrophenol from pNP-laurate as substrate/min under standard assay conditions. The total enzyme activity was expressed in U/ml and specific activity was expressed as U/mg of protein.

2.1.5. Screening of the mutagenic library

Mutagenic library was screened for thermostable mutant lipase using three tier screening method (Ahmad et al., 2008). The three tier screening was carried out as follows. (A) Transformants were initially patched on to LB agar ampicillin petriplates and kept at 37 °C for the growth. Lipase positive clones were then exposed to 60 °C for 30 min followed by cooling. The petriplate containing lipase clones was overlaid with emulsified solution of tributyrin (1%) in agar (0.5%), and screened by formation of zone of clearance around the colonies. (B) Clones obtained in preliminary screening were further screened on 96 well plate. All mutant clones along with Wt were grown in 96 well plates at 37 °C (overnight) in the presence of 0.1 mM IPTG. It was followed by separation of the supernatant from the cells. The supernatant obtained was exposed to 60 °C in thermal cycler for 1 h, followed by cooling at 4 °C for 15 min. The residual enzyme activity was assayed as per standard assay conditions. The color produced by enzymatic reaction was monitored by ELISA plate reader at 420 nm. The reaction mix without enzyme served as blank. Reaction mix containing enzyme without incubation was taken as control (100%). Finally, the tube assay method was carried out by pre-incubating the enzyme supernatant for 1 h at 60 °C, followed by cooling. Enzyme was assayed according to standard assays protocol (C).

2.1.6. Nucleotide sequencing and analysis

To confirm the mutation, the plasmid from the selected mutant clone was sequenced using universal M13 forward and reverse primer. The nucleotide sequencing was done by commercial available service provided by Bangalore Genei (India), using an automated AB1 3100 genetic analyzer, that uses fluorescent label dye terminator, based on dideoxy chain termination method (Sanger et al., 1977).

2.1.7. Expression and purification of the Wt and lip M1

All steps for purification of Wt and lip M1 enzymes were carried out separately at 4 °C, unless and otherwise stated. Both, the Wt as well the lip M1 genes were further sub-cloned in pQE30-UA expression vector, without the terminal signal. One percent overnight grown culture from both Wt and lip M1 was inoculated separately into 500 ml LB containing 30 μg/ml kanamycin and 100 μg/ml ampicillin. The cultures were grown till OD_{600} reached ~0.5–0.6 and recombinant protein expression was induced by addition of 0.1 mM IPTG. After 3 h, the induced culture was pelleted down by centrifugation, at 8000×g for 20 min. Cell pellets obtained were lysed in 40 ml lysis buffer (150 mM NaCl, 0.1% Triton X-100 and 50 mM phosphate buffer, pH 8.0), and kept on ice for 3–4 h. Lysed cell pellets were sonicated with 10 s ON and OFF pulse rate in sonicator, for 10 min. Sonicated cells were centrifuged again at 10,000 rpm for 15 min, to remove the cell debris. The intracellular protein expressed as 6xHis-tag was purified from the soluble fractions, using Ni-NTA resin (QIAGEN, Germany). The fractions showing enzyme activity were pooled and dialyzed against 50 mM NaH_2PO_4, pH 8.0. The protein concentration and enzyme activity of pooled fraction was determined.

2.2. Biochemical characterization

2.2.1. Polyacrylamide gel electrophoresis

The dialyzed protein was analyzed for purity under denaturing condition on 12% SDS-PAGE gel (Laemmli, 1970).

2.2.2. Effect of temperature on enzyme activity and stability

Optimum temperature for the Wt and lip M1 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