Gene 576 (2016) 237–243

Contents lists available at ScienceDirect

Gene

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

Research paper

Disruption of N terminus long range non covalent interactions shifted $temp._{opt}$ 25 °C to cold: Evolution of point mutant *Bacillus* lipase by error prone PCR

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ARTICLE INFO

Article history:
Received 25 August 2015
Received in revised form 17 September 2015
Accepted 5 October 2015
Available online 8 October 2015

Keywords:
Bacillus lipase
Directed evolution
Cold adaptation
Homologue modelling
Sequence alignment

ABSTRACT

Cold adapted enzymes have applications in detergent, textile, food, bioremediation and biotechnology processes. *Bacillus* lipases are 'generally recognized as safe' (GRAS) and hence are industrially attractive. *Bacillus* lipase of 1.4 subfamily are of lowest molecular weight and are reversibly unfolded due to absence of disulphide bonds. Therefore these are largely used to study energetic of protein stability that represents unfolding of native protein to fully unfolded state. In present study, metagenomically isolated *Bacillus* LipJ was laboratory evolved for cold adaptation by error Prone PCR. Library of variants were screened for high relative activity at low temperature of 10 °C compared to native protein LipJ. Point mutant sequenced as Phe19 → Leu was determined to be active at cold and was selected for extensive biochemical, biophysical characterization. Variant F19L showed its maximum activity at 10 °C where parent protein LipJ had 20% relative activity. Psychrophilic nature of F19L was established with about 50% relative active at 5 °C where native protein was frozen to act. Variant F19L showed no activity at temperature 40 °C and above, establishing its thermolabile nature. Thermostability studies determined mutant to be unstable above 20 °C and three fold decrease in its half life at 30 °C compared to native protein. Far UV-CD and intrinsic fluorescence study demonstrated unstable tertiary structure of point variant F19L leading to its unfolding at low temperature of 20 °C. Cold adaptation of mutant F19L is accompanied with increased specific activity. Mutant was catalytically more efficient with 1.3 fold increase in k_{cat}. Homologue structure modelling predicted disruption of intersecondary hydrophobic core formed by aromatic ring of Phe19 with non polar residues placed at β3, β4, β5, β6, αF. Increased local flexibility of variant F19L explains molecular basis of its psychrophilic nature.

1. Introduction

Stabilization strategies adopted by protein under extreme conditions are complex and attributed to various factors. Primary force driving protein folding is entropic encaging of non polar groups away from water molecules and this hydrophobic effect is primary determinant of protein stability. Decreased core hydrophobicity, fewer weak interactions and increased confirmational entropy are the molecular factors for increased activity at cold. Cold adadpted enzymes are desirable because of its increased catalytic efficiency and to minimize the unwanted side chemical reactions. Cold lipases have applications in bioremediation and laundry washing (Choo et al., 1998; Joseph et al., 2008).

Bacillus genus with large and hetrogenous group of bacteria, inhabit some of the most extreme places of earth. These are aerobic, facultative anaerobic, rod shaped endospore forming bacteria. Bacillus lipases are divided into two subfamilies 1.4 and 1.5 in current classification of lipases. Lipolytic enzymes from *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus licheniformis* are grouped in subfamily 1.4 (Eggert et al., 2002). No lid domain is present and hence active site is solvent exposed. They exhibit extreme alkaline tolerance between pH 9.5–12 but are active for limited temperature range of 30–45 °C. There are some metagenomically isolated *Bacillus* lipases reported to be thermophilic (Johri et al., 2012; Zhu et al., 2013) and psychrophilic (Litantra et al., 2013; Wi et al., 2014).There are many reports of laboratory evolution of *Bacillus* lipase for increased thermal tolerance (Acharya et al., 2004; Abraham et al., 2005; Bustos-Jaimes et al., 2010; Khurana et al., 2011; Akubult et al., 2013; Kumar et al., 2013;Sharma et al., 2014). Two approaches are mainly used to alter enzyme properties at genetic level i.e. 1) Directed evolution 2) Rational protein design. Directed Evolution has made dramatic impact in protein study since its introduction by Stemmer in 1994. Directed evolution is natural evolutionary process mimicked at lab bench and does not require target protein structure

Abbreviations: IPTG, isoprpyl-β-thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; CD, circular dichroism; PIC, protein interaction calculator.

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http://dx.doi.org/10.1016/j.gene.2015.10.006
0378-1119/

for evolution as in rational engineering. In present study, *Bacillus lip* metagenomically isolated (accession number FJ5444454.1) encoded lipase of 210 amino acid with 28 residue signal peptide at N terminal region. Its catalytic triad involve Ser77, Asp133 and His156 (position excludes signal sequence). BLAST programme determine *lip* encoded lipase LipJ to share 99% sequence homology with triacylglycerol lipase synthetic construct (ACM61986), esterase of *B. subtilis* subsp. 168 (NP388716.1). Khurana et al. (2011) randomly evolved LipJ variant I56T with enhanced thermostability by directed evolution. In present study *lip* gene was randomly evolved by error prone PCR and variants adapted at cold were screened and selected. Point mutant (F19L) adapted at cold was extensively characterized biochemically, biophysically and by homologue modelling to explain molecular basis of variant behaviour.

2. Materials and methods

2.1. Reagents/Vectors

Metagenomically isolated *lip* gene cloned in pGEMT vector was obtained from lab. Chemicals/Reagents used in the study were of analytical grade, purchased from Merck (Germany), HiMedia (India). Gel extraction kit, pNP substrates were from Sigma Chemicals Co. (USA). Ni-NTA was purchased from Qiagen (Germany). Mutazyme I DNA polymerase for Error prone PCR was obtained from Genmorph random mutagenesis kit from Stratagene. pGEM-T easy vector and pQE30 vector was obtained from Promega (USA) and Qiagen (Germany) respectively.

2.2. Random evolution by error prone PCR

Novel mutant Taq DNA polymerase i.e. mutazyme I DNA polymerase from Genmorph random mutagenesis kit was used to introduce random mutation to *lip* gene. Mutation frequency (number of mutations/kb) depends upon a) Initial amount of target DNA b) Number of PCR cycles. For low range of mutation frequency of 0–4.5 mutations/kb, 500 ng of target DNA is amplified for 20–25 cycles. PCR was carried out at 95 °C for 2 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. PCR product yield was analysed on agarose gel. Gene *lip* was used as template with Fwd primer (5′-GATCGCCCATGGGTGAAAAAA GTACTT-3′) and Rev. primer (5′-GATCGCGGATCCTTAATTTGTATTGAG-3′). PCR amplified mutated library was ligated to pGEM-T easy vector.

2.3. Screening of mutagenic library and selection of cold adapted mutant

Library of mutated variants were spread on LBT-amp plates and screened for expressing ones. Expressing variants were patched on selective plates and incubated at 10 °C. Control plates were kept at 37 °C. Colonies showing larger zone of clearance at cold condition of 10 °C were selected for next tier of screening. Variants with high relative activity at cold were inoculated and cultured in each well of 96 well culture plate. Supernatant was isolated from each clone and was assayed for activity at 10 °C with pNP-laurate as substrate. Colour produced by enzymatic action was monitored by ELISA plate reader at 420 nm. Reaction without enzyme was taken as blank. Results were compared for native protein. Clones showing high relative

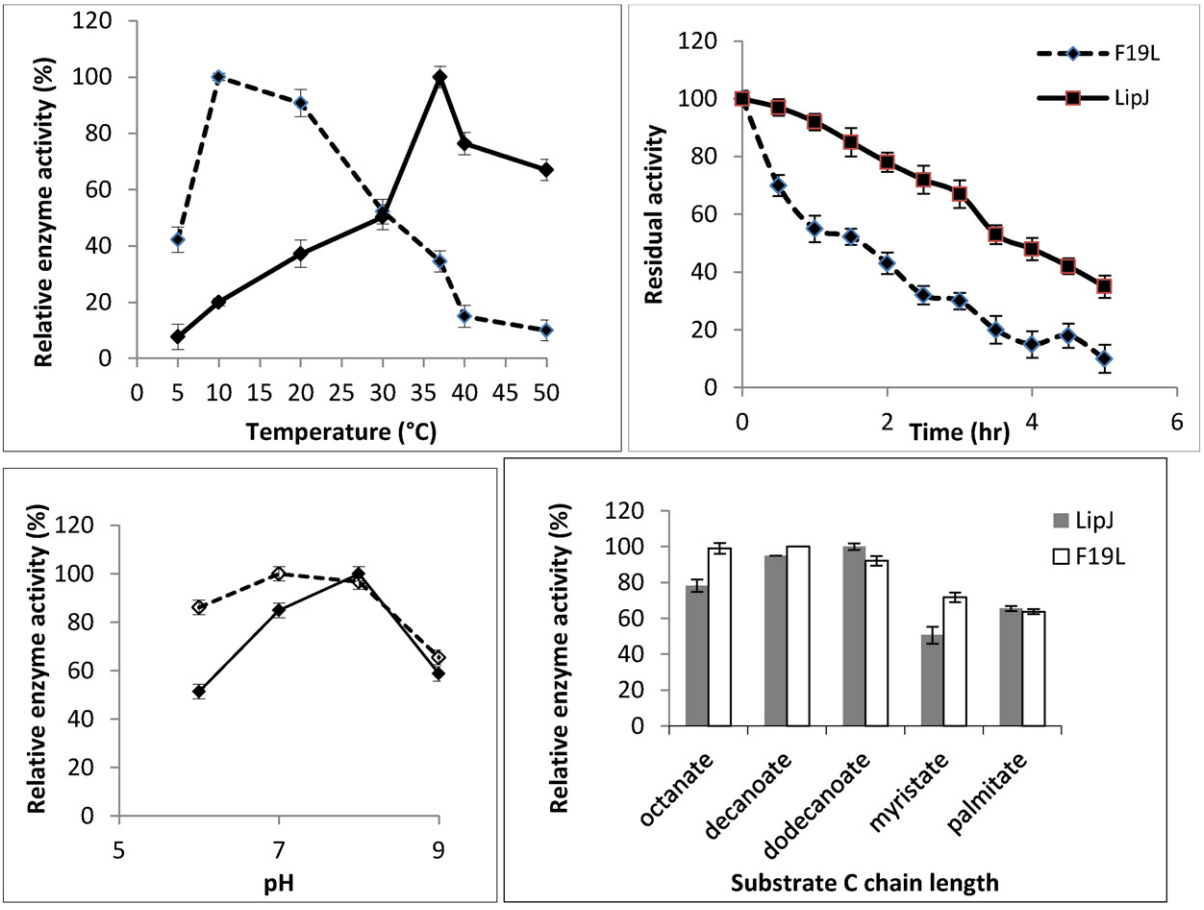

Fig. 1. Biochemical characterization of LipJ and mutant F19L. a) Temperature–activity curve of LipJ and mutant. b) Time course of inactivation of LipJ and F19L at 30 °C. c) Effect of pH on activity of LipJ and mutant. d) Substrate specificity pattern.