Studying Altered Biochemical Properties of a WT Lipase by Mutating a Polar Amino Acid to Non Polar on the Protein Surface.

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ABSTRACT

Mutation altering biochemical properties generally exist close to active site. In the present investigation, a WT lipase gene cloned from a metagenome source revealed more than 90% homology to a lipase of thermophilic Bacillus species. During the biochemical characterization of the WT enzyme, we observed mere differences in the biochemical properties of metagenomic lipase compared to lipase of Bacillus. Multiple sequence alignment of WT lipases to its closest homologues revealed an alteration of amino acid G315E near the active site residue aspartate, present at 317 position of the mature polypeptide. In order to know the possible implication of this mutation, we employed site directed mutagenesis to change the altered amino acid to the conserved amino acid, present in the homologues. During biochemical characterization, we found mutant to be slightly less thermostable than the WT enzyme. However, it displayed high enzyme activity and stability at neutral and acidic pH than the WT enzyme. The mutant enzyme was also able to hydrolyze the short chain fatty acids with a carbon chain length C₃ and C₄ compared to the WT.

INTRODUCTION

The advancement made in protein engineering method offers valuable opportunity for studying structure and function of a protein. These methods can successfully be utilized for exploring the diverse function of localized amino acids in the proteins. Such techniques are routinely being used in academic and industrial laboratories to improve stability, activity or overall performance of enzymes and organisms. Another useful method employed is directed evolution methods are increasingly appears to be the tool of choice for studying relationship between protein structure and function [1-6]. It is also a popular tool for accelerated adaptation of protein functions (e.g., stability, specificity or affinity) in extreme conditions such as unusual temperatures and organic solvents [7-11] as well as for improvement of recombinant protein biosynthesis [12, 13]. Directed evolution has also given rise to altered specificities and activities of enzymes [14-16]. Furthermore, despite of divergent sequences, homologous proteins from different organisms share significant similarity at structural and functional level. Stability of a folded protein is uphold by weak interactions i.e. vander walls interactions, H-bonding, salt bridges, aromatic and disulfide bonds etc. Generally, these interactions get altered at high temperature i.e. 50-60°C, followed by loss of enzyme/protein function [17]. Even alteration of a single amino acid can significantly modulate the structure and function of a protein. Till date, no rule could be specified for the stabilization of a protein [18, 19]. In general, most of the laboratory evolved thermostable enzymes substituted those residues which were already existed in their structural homologues. The amino acid substitution can result in positive/negative or neutral mutation. In this particular study, we have modified a WT polypeptide by altering a amino acid which was already present in the homologous proteins. During characterization, we observed great variation among the catalytic function of the two enzymes. However, the thermostability of mutant protein was observed to be less than the WT, whilst the pH stability was found to be increased. Our recent review article has provided a valuable insight into the replacement of residues on the surface of protein structure.
We had previously cloned a lipase from metagenome source of hot spring soil [20] (Sharma et al. 2012). Recently, we discussed in detail about a single mutant, N355K, which showed enhanced thermostability compared to WT enzyme [21] (Sharma et al. 2012). More recently, we reported characterization of a double mutant, where effect of N355K and E315G was studied together (manuscript under review). In this particular study, we report substitution effect of highly conserved amino acid residues (Glycine) on the biochemical properties of a wild type metagenomic lipase where this residue was altered to glutamic acid.

MATERIALS AND METHODS

Reagents/kits/Plasmids:
pGEM-T easy vector used for the cloning purpose was purchased from Promega (USA), and expression vector pQE30-UA plasmid was purchased from Quiagen (Germany). Gel extraction kit was purchased from MO BIO (USA). Taq DNA polymerase (5U/µl), dNTPs mix, each was purchased from Fermentas (Germany). Substrates (pNP-esters and tributyrin), used for biochemical assays and screening, were purchased from Sigma Aldrich (USA). All other chemicals were procured from Merck (Germany).

Mutagenesis and subsequent molecular manipulations
Site directed mutagenesis was carried out by overlap extension PCR method. The oligonucleotides used for the site directed mutagenesis were designed by manual method, using IDT tools. The point mutation was introduced at the centre to enhance the stabilization of the non-complimentary interactions during PCR amplifications. The oligonucleotides used were as follows, 5'-TGATGAARGGG NTGYAGRGTNCC-3' (forward) and 5'-TGATGAARGGG NTGYAGRGTNCC-3' (reverse) for WT gene cloning. The oligonucleotides used for the amplification of a mutant gene was as follows, 5'-ATTGGCTTG GGAACGCGG-3' (forward) and 5'-CCGTGGTCCCAGCCTTGC-3' (reverse). A gradient PCR was performed in a Bio-Rad thermal cycler as follows: 94°C for 4 min, followed by 30 cycles at 94°C for 1 min, 55/59.5°C for 50 seconds and 72°C for 2 min, with a final extension of 10 min at 72 °C. The amplified mutated gene fragment was cloned in pGEM-T easy vector, as described by the manufacturer. Mutation was confirmed by sequencing followed by its submission in the gene data bank. Furthermore, genes encoding WT and mutant E315G were sub-cloned in pQE-30 UA expression vector, and transformed in E. coli M15 cells. Transformed cells were selected on LB agar medium plates containing ampicillin (100 µg) and kanamycin (35 µg).

Protein expression and purification
E. coli M15 cells harbouring recombinant pQE-UA-lipase (WT and mutant) genes were grown overnight at 37°C in 5 ml liquid LB medium containing ampicillin (100 µg/ml) and kanamycin (30 µg /ml) overnight. Next day, sub culturing was done the adding 1% overnight grown cultures into 500 ml LB media. The culture was grown at 37°C till OD600 was reached to ~0.5. The expression was induced by addition of 0.1 mM. Isopropyl-beta-thio galactopyranoside (IPTG) in the culture. Cells were harvested after four hours of the induction and the clear supernatant was collected. The protein secreted out of the cells was purified from the supernatant as described previously [22]. All steps for purification of WT and mutant proteins were done separately and carried out at 4°C. Protein concentration was determined using the commercially available BCA (Bicinchoninic acid method) kit (Merc, Germany). Bovine serum albumin was used as standard. Protein Samples were analyzed on SDS– PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) according to Laemmli [23] for determining the purity and molecular mass of proteins.

Enzyme assay
All enzyme assay for the lipase activities were performed according to Sigurgisladottir et al. [24]. The 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 per min under standard assay conditions. The total enzyme activity was expressed in Unit/ml and specific activity was expressed as Unit/mg of protein.

Biochemical properties:
Temperature optimum for the WT and mutant enzyme was determined by assaying purified enzyme at wide range of temperature i.e. from 20°-80°C. Thermal denaturation of enzymes was studied at these temperatures by pre incubating enzymes at this temperature for 30 min. The thermostability assays were further carried out at 50 and 55 for 75 minutes by pre-incubating enzyme at these temperatures. The enzyme aliquots were removed and assayed after every 15 minutes using standard assay conditions followed their cooling in ice for 5 minutes, before the enzyme assay. The enzyme without incubation was taken as control (100%). The pH optimum for the WT and mutant enzyme was determined by assaying enzymes at wide range of pH i.e. KCl-HCl (pH 1.6-2.0), Gly/HCl (pH 2.5), Citrate buffer (pH 3.0-6.0) Sodium phosphate buffer (pH 6.5-8.0), Tris. HCl (pH 8.5), Gly/NaOH (pH 9-10.5).
All buffers were prepared in the Milli-Q water, as 0.5M stock and diluted appropriately whenever required. Enzyme stability was assayed by preincubating appropriately diluted enzyme in 50 mM buffer of different pH (pH 8.0) at room temperature. The enzyme assays were performed according standard assay conditions. The enzyme without incubation was taken as control (100%). Substrate specificity of the WT and mutant lipase was determined using pNP ester of following chain length, pNP-acetate (C₃), pNP-butyrate (C₄), pNP-caprylate (C₈), pNP-deconate (C₁₀), pNP-laurate (C₁₂), pNP-myristate (C₁₄), pNP-palmitate (C₁₆), pNP-stearate (C₁₈) (Sigma USA), dissolved as 10 mM stock in ethanol and were finally used as 2 mM concentration. Biochemical kinetics of WT and mutant was determined, over range of substrate concentration from 0.01 mM-2.5 mM (pNP laurate). The Michaelis–Menten constant (Kₘ) 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/K_m were also calculated and the results were compared.

Secondary structure determination and calculation of the pI:
Secondary structure for the mature polypeptide in case of wild type and mutant was calculated by by SOMPA and the pI of the mature polypeptide was calculated using ProtParam.

RESULTS AND DISCUSSION

Mutagenesis, molecular manipulation and recombinant protein purification
Site directed mutagenesis was carried out to introduce mutation E315G in the WT lipase gene, as stated in materials and methods. The PCR reaction initially amplified partial length gene fragments at 231 bp and 1038 bp (Fig. 1a). The overlap extension PCR carried out by using these partially amplified DNA fragment as template resulted in amplification of the full length gene fragment ~1260 bp along with the non specific amplification at 350 bp (Fig. 1b). The specific band was gel eluted, ligated with the cloning vector pGEMT and transformed into E. coli DH5α. The recombinant clones were selected on LB agar plates having emulsified tributyrin (Fig. 1c). Subsequently, the gene was cloned in pQE-30 UA expression vector showed expression on inducing with the addition of 0.1 M IPTG. The over-expressed protein was purified extracellularly from WT and mutant lipase was checked on 12% SDS-PAGE (Fig. 2). Mutant enzyme showed ~ 4 folds increase in the purification and % yield ~5 times high compared to WT enzyme (data not shown). The specific activity of the mutant and WT was determined to be 2022±3 U/mg protein1816.2±18 U/mg respectively.

Biochemical characterization
Both the mutant and WT enzymes were found active over wide range of temperature (35-50 °C). The optimum temperature for the mutant was calculated to be 50 °C and was comparable to the WT (Fig. 3a). The enzyme was active over wide range of pH and displayed optimum activity at pH 9, which was comparable to the WT enzyme (Fig. 3b).
Fig. 2: SDS PAGE (12%), Lane 1&7: control sample, Lane 2&6: IPTG induced sample after 4h growth, Lane 3&5: Purified protein sample from mutant and WT respectively, Lane 4: Protein molecular weight marker

Fig. 3a

Fig. 3b

Fig. 3c

Fig. 3d

Thermal stability assays

The stability of the mutant was evaluated for different time intervals, at 50 and 55°C. At 50°C WT enzyme was found to be stable for 60 min whereas the mutant enzyme showed less thermostability after 30 min. Further incubating protein for 75 min, the WT showed more than 70% enzyme activity, whereas the mutant showed ~40% enzyme activity (Fig. 3c). We also compared thermal stability of WT and mutant at 55°C (Fig. 3d). In our previous
study, we have shown that mutation N355K on the protein surface resulted in 144 folds enhancement in the protein thermostability of the mutant compared to the WT protein [22], in present study the mutation E315G did not showed much alteration in function of protein.

Substrate Specificity
We tested the effect of substrates (pNP ester) of different chain lengths on enzyme activity and found that both mutant and the WT has 100% relative enzyme activity with C_{12}, whereas, with C_8 and C_{10}, mutant displayed 85 and 90% relative enzyme activity, while WT showed less than 10% relative enzyme activity. Interestingly, the mutant also showed activity towards C_3 and C_4, whereas with WT, no enzyme activity was observed towards these substrate. No significant differences were observed between WT and mutant enzymes towards C_{14}, C_{16} and C_{18}. The results demonstrated the order of substrate specificity as follows C_{12}>C_{10}>C_8>C_{14}>C_{16}>C_{18}>C_3 (Fig. 4). Enzyme activity of the lipase as a function of substrate concentration was carried out.

Secondary structure prediction:
To shed light into the change in the secondary structural contents, we predicted structure of the two proteins using software SOMPA available at Expasy site, the data for the mutant revealed that the protein consisted of 23.20% \( \alpha \)-helix, 8.5 % \( \beta \)-turn, 21.3% extended coiling and 47.16% random coiling, whereas the WT protein has 25% \( \alpha \)-Helix, 20.36% extended strand, \( \beta \)-turn 8.76% and random coiling 45.88%. From the secondary structural predictions, it become evident that though there is minor difference in secondary structural contents among the two, it seems to be significant. Moreover, there is very much possibility of structural perturbations on bringing glycine (a flexible amino acid) in place of glutamic acid in the polypeptide chain, which might result in alteration of the structure near binding site of the substrate.

REFERENCES

Available online at www.scholarsresearchlibrary.com