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Enhancing activity and thermostability of lipase A from *Serratia marcescens* by site-directed mutagenesis

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ABSTRACT

Lipases as significant biocatalysts had been widely employed to catalyze various chemical reactions such as ester hydrolysis, ester synthesis, and transesterification. Improving the activity and thermostability of enzymes is desirable for industrial applications. The lipase of *Serratia marcescens* belonging to family I.3 lipase has a very important pharmaceutical application in production of chiral precursors. In the present study, to achieve improved lipase activity and thermostability, using computational predictions of protein, four mutant lipases of SML (MutG2P, MutG59P, Mut H279K and MutL613WA614P) were constructed by site-directed mutagenesis. The recombinant mutant proteins were over-expressed in *E. coli* and purified by affinity chromatography on the Ni-NTA system. Circular dichroism spectroscopy, differential scanning calorimetry and kinetic parameters (Km and kcat) were determined. Our results have shown that the secondary structure of all lipases was approximately similar to one another. The MutG2P and MutG59P were more stable than wild type by approximately 2.3 and 2.9 in $T_{1/2}$, respectively. The catalytic efficiency (kcat/Km) of MutH279K was enhanced by 2-fold as compared with the wild type ($p < 0.05$). These results indicate that using protein modeling program and creating mutation, can enhance lipase activity and/or thermostability of SML and it also could be used for improving other properties of enzyme to the desired requirements as well as further mutations.

1. Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are serine hydrolases that catalyze both the hydrolysis and the synthesis reactions [1]. Moreover, microbial lipases do not require cofactors and possess broad substrate specificity, hence are widely used in a variety of industrial applications such as the synthesis of fine chemicals and optically active pharmaceuticals [2]. Lipases have been widely produced by microorganisms, mainly bacterial, and are commercially important due to their diversity in catalytic activity, high yield and low cost production, as well as the relative ease of genetic manipulation [3,4]. Bacterial lipases based on the difference in amino acid sequence and biochemical properties classified into eight different families (I–VIII). Family I, being the largest group and is further divided into seven subfamilies (I.1–I.7) with the first three subfamilies (I.1–I.3) including of gram-negative bacteria true lipases [5,6]. Subfamily I.1 and I.2 lipases share relatively high amino-acid sequence similarity, in-contrast subfamily I.3 lipases possess low amino-acid sequence similarity (<20%) to either family I.1 or family I.2 lipases [7]. In the other hand, those have higher molecular size than lipases from subfamilies I.1 and I.2 and both the lack of Cys residue [8]. Family I.3 lipases, which are represented by lipases from *Serratia marcescens* (SML) and *Pseudomonas fluo-*

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rescens, are separated from other lipases not only by their amino acid sequences but also by their secretion mechanisms and biological properties [9]. The SML is composed of 613–614 amino acid residues with a molecular weight between 64 and 65 kDa [10,11]. The lipase A from *Serratia marcescens* has been the subject of research for more than 30 years, as it is used for production the large scale kinetic resolution of racemic 3-(4-methoxyphenyl) glycidic acid methyl ester which it is a chiral precursor for diltiazem synthesis, a calcium-channel blocker and coronary vasodilator [12]. In recent years, many efforts have been made to improve lipase activity, and in particular, to enhance lipase stability. Various strategies to protect lipases from inactivation and to increase the operational stability and activity of lipases have been developed, including the use of chemical modification of structure, protein engineering, medium engineering, etc [13]. Many factors are that influence enzyme stability, which of all potentially deactivating factors, temperature is the best studied [14]. In order to use lipases as biocatalysts in industrial applications, it is desirable improving properties such as the thermostability and activity, that prolongs product shelf half-life, increase energy efficiency, and save costs hence, there is great interest to generate enzymes with desired properties [15–17]. Nevertheless to achieve the above objective, requires the use of the modern methods of genetic engineering combined with an increasing knowledge of the structure and function of lipases [18,19]. Thermostable lipases are more attention for industrial processes because the reactions might be performed at elevated temperatures therefore should be the structure of enzyme in harsh condition is maintained, furthermore includes other advantages such as increased solubility of lipid substrates in water, faster reaction and reduced possible risk of contamination [20,21]. To improve one or more properties such as the thermostability and the activity of the enzyme, widely used strategy include hydrogen bonding, the formation of disulfide bridges, hydrophobic or aromatic interactions, ion pairing, etc [22]. Therefore, to achieve these goals a suitable and efficient method must choose. For this purpose, there are two major and principally different routes: (i) the first, structure-based rational design, in here requires to structural information and knowledge about three-dimensional (3D) of protein. Using this method can predict and improved the activity and stability of enzyme, for example, the site-directed mutation (SDM) technique here is used [23–26]. The second, (ii) random or "irrational" mutagenesis design, relying on entirely random mutagenesis techniques by various protocols for generating large variant libraries of genes, followed by screening or selection for improved variants for specific properties of an enzyme, such as utilizing the gene (DNA) shuffling and error-prone PCR (epPCR) technique. In here for directed evolution usually does not require prior knowledge of the structure of the enzyme (protein) [27–32]. The fact that the rational protein design methods through computational models have been developed and it have provided a new valuable tool for improving enzyme properties to the desired requirements [33]. This method has experienced important with varying degrees of success in recent years, with considerable achievements in the design of novel enzymes [34–36]. Structural features of an enzyme can affect the thermostability as well as plays a crucial role in promoting thermostability. For example, thermostability is associated with large numbers of electrostatic interactions, a large number and tight metal-binding sites, high packing density and core hydrophobicity. Thus, a rational approach (e.g. site-directed mutagenesis) can be used in the presence of empirical testing to enzyme engineering as well as optimizing the activity and thermal properties of an enzyme [37]. Understanding the structural basis of altered properties of proteins due to changes, provides useful insight in designing proteins with improved catalytic activity or thermostability. We describe here progress in improving the stability and activity of SML by rational site-directed mutagenesis (SDM) based on homology modeling and molecular dynamics (MD) simulations as well as experimentally testing. In this study, four mutant proteins of SML were designed and constructed, and was analyzed enzyme activity and thermostability.

2. Materials and methods

2.1. Bacterial strains, plasmids, enzymes, materials and culture conditions

Marine *Serratia marcescens* (GenBank No. GQ471957), which is deposited into the Microorganisms collection of the Microbial Technology and Products (MTP) Research Center of Iran (Collection No. UTMS 2342), was utilized for experiments. *Escherichia coli XL1* −Blue [recA1 *endA1 gyrA96 thi-1 hsdR17 supE44 [F' proAB lacI^{q}ZΔ15 Tn10 (Tet^r)*] (Novagen-USA) and *Escherichia coli BL21* (DE3) [*F$^-$ ompT hsdSB (rB$^-$ mB $^-$) gal dcm* (*lcIts857 ind1 Sam7 nin5 lacUV5-T7 gene1)*] (Novagen-USA) were employed as host strains for the gene manipulation and protein expression, respectively. The plasmids used for the cloning and protein expression was pUC19 Amp^r and pET-28a (+) Kan^r (Novagen-USA). All the restriction enzymes were from Thermo Scientific (USA), all the kits were purchased from Qiagen (USA)/Roche (Germany). All other chemicals and reagents used in the study were of good analytica grade, obtained from Merck (Germany)/Sigma Aldrich (USA). The *S. marcescens* was grown in a nutrient broth (0.3% beef extract, 0.5% peptone and 0.5% NaCl) at 30 °C. LB medium (1% peptone, 0.5% yeast extract, 1% NaCl) or solid medium contained bacto-agar (1.5%) were used for culture and growth of *E. coli* at 37 °C. Ampicillin (100 μg/ml) and Kanamycin (50 μg/ml) were added to medium for recombinant *E. coli* when needed. Isopropyl β-1-D-thiogalactopyranoside (IPTG) was used at indicated time point for induction of lipase production when needed. The cell growth was determined by measuring the optical density at 600 nm using a spectrophotometer.

2.2. Construction of wild-type expression plasmid

The genomic DNA of *S.marcescens* was extracted by the bacteria genomic DNA extraction kit (Roche Germany). The SML gene *(lipA)* (accession no. KF372589) of *S. marcescens* was amplified using Pwo DNA polymerase (Roch) according to the procedures recommended by the Supplier in combination of primer 1 (5′-GCCCAT**ATG**GGCATCTTTAGCTATAAGG-3′), primer 2 (5′- TAGGATCC**TTA**GGCCAACACCACCTGATC-3′) where, the underlined bases represent the *Nde*I site for primer 1, and *Bam*HI site for primer 2. The ATG codon for the initiation of the translation and the sequence complementary, TAA, to the termination codon are shown in bold type. The PCR products were digested with with *Nde*I and *Bam*HI, purified, and ligated into the *Nde*I and *Bam*HI sites of pET-28a(+) vector and was then transformed into XL-1 Blue cells for cloning and verification through sequencing. The verified pET28a-LipA was transformed into *E. coli* BL21 (DE3) for overexpresssion of the lipA gene.

2.3. Homology model

2.3.1. Computational design of mutants

In order to identify potential mutations that would lead to enhanced catalytic activity and thermostability, studied SML structure was modelled using the homology model module of MOE 2012.10 based on the existing crystal structures of SML (PDB code 2QUA). Identification of putative mutations was performed based on two approaches: comparison of SML structure with *Pseudomonas* lipase (PML) (PDB code 2Z8X) and direct investigation on SML structure itself. In order to evaluate catalytic activity of SML, the obtained SML model was superimposed on PML structure and