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Development of a catalytically stable and efficient lipase through an increase in hydrophobicity of the oxyanion residue

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ABSTRACT

In-silico and empirical site-directed substitutions of oxyanion Q114 of the wildtype T1 lipase with that of hydrophobic Leu and Met residues were carried out to afford the Q114L and Q114M lipases, respectively. Using the esterification production of menthyl butyrate as a reaction model, evaluation on the catalytic efficiency of the three lipases was performed. It was found that Leu evidently improved the catalytic activity of the Q114L lipase, achieving the highest conversions of menthyl butyrate under varying experimental conditions that may be attributable to its lower total energy when compared with both the T1 and Q114M lipases. The diminishing catalytic activity of T1 lipase observable following substitution with Met (Q114M lipase) may be due to formation of three additional cavities within the vicinity of the mutation, lesser density of the protein core and susceptibility to high temperature, particularly under prolonged reaction time. Therefore, it can be concluded that the substitution of Leu into the oxyanion-114 had rendered favorable structural changes that enhanced the catalytic activity of the T1 lipase, envisaging that such approach may also be of applied value for improving the catalytic activity of other bacterial lipases within the I.5 family.

1. Introduction

Hydrophobicity as well as high density of residues within the interior protein have been suggested as major factors affecting stabilization of protein structure [1,2]. A compromise between close packing and conformational strain to form a dense protein core packing is often required to overcome energetically unfavorable perturbations [3]. Such a compromise would lead to formation of cavities especially among large proteins [4,5], an inevitable aspect in protein architecture [1]. This is particularly true for folded proteins such as enzymes which hydrophobic amino acids are generally located deep within the protein for efficient packing [6]. It has been suggested that it is possible that he stability of the protein interior against various denaturants with a single amino acid substitution [7] that increases the hydrophobicity of the protein core. However, possibility of expansion in the size and increased number of cavity within the hydrophobic core (i.e., defects in atomic packing) may counteract the benefits of such modifications and destabilize the protein molecule [8].

Lipases (triacylglycerol hydrolases E.C.3.1.1.3) are industrially important enzymes that catalyze a broad range of reactions such as esterification, *trans*-esterification, organic synthesis under water-restricted environment, and stereospecific hydrolysis of racemic esters. Many efforts have been made to further optimize their enzymatic properties through advances in site-directed mutagenesis

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and directed evolution [9]. In view of elucidating the possible role of amino acid sequence in stability of protein in lipases, molecular approaches that explore the effect of amino acid substitution on conformational stability of mutant proteins have been suggested [10]. Molecular approaches such as site-directed mutagenesis that occurs within the 11 Å distance from active site may alter enantioselectivity of the molecule more strongly than that of distant mutations [10]. Since such mutation may substantially change the topology of catalytic pocket and subsequently affect the stability as well as efficacy of lipase-catalyzed reactions; improved tolerance of lipases towards the different solvents, high temperatures, and denaturants as well as enantioselectivity may possibly be obtained [10–13]. Therefore, developing potentially more expedient approaches for creating new catalytically more stable and efficient lipases through protein mutation may prove useful.

Previously, we reported on a lipase called T1 produced by the thermoalkalophilic *Geobacillus zalihae*. The lipase gene encoding 388 amino acids residues using vector pGEX 4-T1 was highly expressed in recombinant *Escherichia coli* BL21 (DE3) pLys*S*. The catalytic machinery is of T1 lipase consists of a triad of amino acids formed by Ser-114, His-359 and Asp-314 [14]. The oxyanion of T1 lipase was deduced to be Q114 and F16, based on another well-known thermoalkalophilic lipase called BTL2 lipase of the *Bacillus thermocanulatus*. The BTL2 lipase has been successfully crystallized in open conformation which also revealed that residues Q115 and F17 formed the oxyanion of the enzyme. The F17 residue in BTL2 lipase was reportedly to be highly conserved [15], hence, it was as expected of the F16 in T1 lipase. More importantly, the BTL2 lipase shares a high sequence similarity to the T1 lipase, thereby, serves as an excellent reference template for the in-silico mutational studies. In this paper, site-directed mutagenesis on the oxyanion Q114 in the protein core of the wildtype T1 was performed whereby the hydrophilic Gln was substituted with hydrophobic, Leu and Met for producing variants Q114L and Q114M lipases, respectively. During in-silico investigations, FoldX and Voronoia 1.0 were used for predicting the conformational changes on the Q114L and Q114M structures and compared with that of wildtype T1 lipase. In addition, an empirical study for comparing the catalytic efficacy of lipases Q114L and Q114M with that of the wildtype T1 lipase was carried out using solvent-free esterification production of menthol butyrate as a reaction model. The efficiency of lipases Q114L, Q114M and T1 were systematically investigated under a variety of experimental conditions that included incubation time, temperature, enzyme amount, substrate molar ratio and agitation speed.

2. Materials and methods

2.1. Materials

The components for the growth media for the lipases were purchased from Difco Laboratories (Detroit, USA). Menthol and butyric anhydride was purchased from Sigma–Aldrich (St. Louis, USA), and HPLC grade isooctane was obtained from Merck (Darmstadt, Germany). Antibiotics, chloramphenicol, and ampicillin were acquired from Amresco (Ohio, USA). Glutathione-Sepharose HP, Sephadex G25 were from GE Healthcare (Buckinghamshire, United Kingdom). Dithioreitol (DTT), phosphate buffer (pH 7.0 and pH 7.4), sodium hydroxide (NaOH), buffer Tris–HCl (pH 8.0), NaCl and $CaCl_2$ were purchased from Sigma–Aldrich (St. Louis, USA). Amresco assay reagent and bovine serum albumin (BSA) were also procured from Sigma–Aldrich (St. Louis, USA). Amicon Ultra-15 centrifugal and 0.45-μm membrane filter (Sartorius) filter were purchased from Millipore (Bedford, USA) and Sartorius (USA), respectively. Distilled and deionized water were produced in our laboratory.

2.2. Computational studies: in-silico mutation, calculation of structural stability and protein compactness

In-silico protein mutation and estimation of protein stability were performed using FoldX Version Beta 3.0. The crystal structure of T1 lipase (2DSN) of the thermoalkalophilic lipase from *G. zalihae* was retrieved from PDB file and the structure was repaired using FoldX for correction of residues with bad torsion angles. For accuracy, the presence of crystal water and metal within the protein structure were also taken into consideration. The three dimensional model of each lipase variant was constructed by the software and possible conformations of each residue following mutation were analyzed. Each lipase variant was subjected to simple energy minimization to remove high energy local minima. Finally, calculation of individual protein stability was performed and compared to the wild-type T1 lipase. The standalone program downloaded from the website http://bioinformatics.charite.de/voronoia [16] was used to calculate the packing density and total cavities of each lipase mutant. The Protein Data Bank files of the wildtype T1, Q114L and Q114M lipase, previously constructed in FoldX were submitted to the Voronoia 1.0 standalone program which calculated for the packing density and total cavities of each lipase.

2.3. Preparation of working culture and purification of enzyme

The wildtype T1 lipase is from a thermoalkalophilic lipase called *G. zalihae* (2DSN) previously isolated from palm effluent. For the working culture, the Q114L, Q114M and wildtype T1 strains were revived from stock culture and grown in Luria Bertani (LB) broth supplemented with antibiotics ampicillin (50 μg/mL) and chloramphenicol (35 μg/mL). The cultures were centrifuged (10,000 rpm, 10 min), the liquid decanted and the pellet was re-suspended in distilled water prior to sonication (Branson 250 sonifier: output 2, duty cycle 30 and min 2). The cell lysate was cleared by centrifugation (12,000 rpm, 20 min) and the supernatant collected. The pH of supernatant was adjusted to pH 9.0 using NaOH (2M), lyophilized and stored in −20 °C until further use.

For the purification process, the cultures containing Q114L, Q114M and wildtype T1 lipase (1000 mL) were harvested by centrifugation, re-suspended in 40 mL phosphate-buffered saline (PBS; pH 7.4) containing 5 mM DTT, and sonicated (Branson 250 sonifier: output 2, duty cycle 30 and min 2). The cell lysate was cleared by centrifugation at 12,000 × *g* for 30 min and filtered with a 0.45-μm membrane filter. Glutathione-Sepharose HP (10 mL) was packed into an XK 16/20 column and was equilibrated with 10 column volumes of PBS. The cleared cell lysate was loaded on the glutathione-Sepharose HP column at a flow rate of 0.25 mL/min. The column was washed with phosphate buffer solution (pH 7.0) until no protein was detected. The bound lipase was eluted with a buffer containing 100 mM Tris–HCl, 100 mM NaCl, and 0.33 mM $CaCl_2$, pH 8.0. The enzyme-containing fractions were determined by SDS-PAGE, pooled, and concentrated using Amicon Ultra-15 centrifugal filter and was tested for lipase activity. The concentrated solution was subjected to gel filtration on Sephadex G25 in an XK16/20 column. The fractions containing lipase activity were collected and concentrated with an Amicon Ultra-15 centrifugal unit. The homogeneity of the partially purified protein was confirmed by SDS-PAGE. The protein was lyophilized and stored at –20 °C.

2.4. Standard lipase activity assay and protein concentration

Lipase activity was determined according to a previously described method by Leow et al. [14] using an emulsion of olive oil