Effect of mutation at oxyanion hole residu (H110F) on activity of Lk4 lipase

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**Abstract**

Mutant of lipase at oxyanion hole (H110 F) was constructed. The gene was highly expressed in Eschericia coli BL21 (DE3) and the recombinant protein was purified using Ni-NTA affinity chromatography. The activity of mutant enzyme was significantly increased compared to that the wild type. Further comparison showed that both of the enzymes exhibited same optimum pH and temperature, and showed highest lipolytic activity on pNP-decanoate (C10). The wild type appeared lost of activity on C14 and C16 substrates meanwhile the mutant still showed activity up to 20 %. In the presence of non polar organic solvent such as n-hexane, the wild type became inactive enzyme meanwhile the mutant still remained 50 % of its activity. The results suggested that mutation at oxyanion hole (H110 F) caused enzyme-substrate interaction change resulting on elevation of activity, better activity toward longer carbon chain substrate and improving the activity in the present of non polar organic solvent.

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1. Introduction

Lipase is an enzyme catalyzing carboxyl ester bonds formation or its hydrolysis. In presence of water the enzyme catalyze carboxylate ester bonds hydrolysis to produce free fatty acids and organic alcohols. Meanwhile in low water environment, the enzyme shows esterification or transesterification activity such as alcoholysis, acidolysis, aminolysis and interesterification [1,2]. Lipase shows important role in many industrial applications such as additive in biodetergent, pulp and paper industries, leather and food industries, fine chemical synthesis, biofuels and biodegradable polymers productions [3–5].

Lipase shows similar structure with other hydroases (esterase, protease, dehalogenase etc) called α/β hydrolase fold that contain eight β pleated sheets surrounded by α helices. The enzyme consists some important structural features such as catalytic triad, oxyanion hole, and lid. Catalytic triad of lipase consists of one nucleophilic (serine), one catalytic acid (aspartic or glutamic residue) and one of histidine residues [6,7]. The enzyme also showed a pocket called oxyanion hole that has role in stabilization of negative oxygen carbonyl group of substrate during intermediate formation. Oxyanion hole consists two residues, the first residue is located between β-strand and αA-helix. The second residue is located at pentapeptide motive on the C-terminus of catalytic residue of serine [8,9]. Highly conserved pentapeptide motive shows pattern G-X-S-A-G, X refers to second oxyanion residue and S refers to serine as nucleophilic residue [10]. Lid has a role of covering active site of the enzyme in the absence of lipid-water interface. While in the presence of interface, lid will open and the substrate interact to the catalytic site [11,12].

Catalytic activity of lipase might be improved by mutation on the catalytic pocket of oxyanion hole [9,13]. There are a few reports showed that mutation on oxyanion hole residues affect features of various lipase [14–18]. Thermostable lipase from G. saliba contains two oxyanion hole residues, Q114 and F16. Mutation on Q114 L resulted on mutant enzyme with better properties such as increasing on optimum temperature, stability in organic solvent and surfactant resisitency [16]. Mutation at oxyanion hole (F146 I) of Yarrowia lipolytica lipase caused on changing in substrate binding and catalytic efficiency [17]. F17S mutation on B. thermocatenolatus lipase exhibited higher activity in the presence of organic solvent [18].

Thermostable lipase were currently highly explored since the enzyme might increase productivity due to increasing reaction rate...
at high temperature. Exploration of thermostable lipases were extensively carried out [19]. Various lipases were isolated from local strains and traditional compost [20–24]. One of the lipase gene namely _Lk_ITB5a_ (Lk4) was previously isolated directly from compost using metagenomic approach at thermogenic phase. The gene consists 936 base pairs coding for 311 amino acids. The lipase has 99% similarity with lipase from *Pseudomonas stutzeri* [A1D66451.1]. Nucleotide sequence of Lk4 was deposited into GenBank with accession number of KP204886 [21]. The lipase has three catalytic triads (Ser109, Asp255 and His277) and two oxyanion hole (Met43 and His110) residues. This paper reported the first mutation in one of oxyanion hole (H110 F) on *Pseudomonas stutzeri* Lk4 lipase that significantly increasing (4 times) the activity of the enzyme.

2. Methods

2.1. Computational method

The 3D structure of proteins was constructed by using SWISS MODEL program (https://swissmodel.expasy.org/interactive) [25]. Lk4 has closest identity with PDB ID 1ex9, which further used as template for 3D structure protein construction. The proteins was visualized by using UCSF Chimera [26]. Ligand (nPND) was created and energy-minimized using Marvin Sketch (https://chemaxon.com/products/marvin). Molecular Docking was performed using Autodock Vina [27]. Gridbox was set as follows (center_x = 0.055, center_y = –2.765, center_z = 45.894, size_x = 12, size_y = 14, size_z = 10). Liggplot+ used to visualize ligand-proteins coordinate after docking process [28]. Interaction between ligand and Lk4/Lk4H110 F also visualized using PLIP (https://projects.biotec-tu-dresden.de/plip-web/plip) [29]. Standalone Voronoiia software was used to estimate total cavities of proteins [30].

2.2. Construction of mutant

The wild type of lipase gene from pJET-Lk4 plasmid was amplified using Feksp and Reksp primers. The mutant was constructed by PCR-site directed mutagenesis using primers namely Feksp (5’-CAACATAGAAGAAACAAACACTGTGCTGCC-3’), Reksp (5’-AAATCGACGAGGCCCCGCTTCTT-3’) [31] and RF3 (5’-GTCGGTCCGGGAGCTTGGGCCG-3’). The first PCR process using Feksp and RF3 primers produced 348 bp amplicon. The amplicon was used as mega primer paired with Reksp primer for the second PCR process to get whole mutant gene. Following confirmation of the mutation, the mutant gene was inserted in the pET30a(+) resulting on recombinant plasmid pET-Lk4H110 F. The recombinant plasmid was used to transform _E. coli_ BL21 (DE3).

2.3. Heterologous expression of protein

Transformant cells were inoculated in LB broth contain kanamycin 50 µg/mL and incubated at 37 °C, 150 rpm, overnight. The culture was inoculated to fresh LB broth and incubated at 37 °C, 150 rpm until OD600 was 0.6–0.8. IPTG was added until final concentration 1 mM. Protein expression was performed at 37 °C, 150 rpm for 4 h. Pellet cell was harvested by centrifugation at 4500 g for 10 min. The protein was extracted using SDS-heating method as follows. The pellet was added with lysis buffer (sodium phosphate buffer 0.05 M pH 8 contain SDS 0.1 % v/v) and shaken with BenchRocker 2D for 30 min at room temperature. Crude extract obtained by lyse the cell suspension at 60 °C for 10 min. Supernatant was separated from cell debris by centrifugation at 12,000 g for 30 min. Supernatant was incubated for 30 min at 60 °C to remove proteases

2.4. Purification of recombinant protein using IMAC Ni-NTA

Purification was performed by gravity flow method at room temperature. 4 ml Ni-NTA agarose suspension was filled into a column. The column was then washed with 3 × 10 ml milliQ water. Binding buffer (sodium phosphate buffer 0.05 M, pH 8, NaCl 100 mM and Triton X 0.1 % v/v) of 3 × 15 ml were passed into the column. A protein solution (∼ 45 ml from ∼1.5 g cell) was passed into the column. The resin was washed with a buffer (sodium phosphate buffer 0.05 M pH 8, NaCl 100 mM) of 2 × 40 ml. The bound proteins were eluted using elution buffer gradually containing 10 mM imidazole (sodium phosphate buffer 0.05 M pH 8, NaCl 300 mM, and imidazole 10 mM), 10 ml elution buffer 80 mM imidazole (sodium phosphate buffer 0.05 M pH 8, NaCl 300 mM, imidazole 80 mM) and 4 ml elution buffer containing imidazole 150 mM (sodium phosphate buffer 0.05 M pH 8, NaCl 300 mM, imidazole 150 mM). Eluted proteins with 80 mM imidazole was dialylated using Amicon Ultra Centrifugal Filter (with 10 kDa cut off membrane) until imidazol concentration was ∼ 0.002 mM.

2.5. Lipolytic assay and characterization

Lipase activity was determined by modified colorimetric method using p-nitro-nitrophenyl fatty acids as substrate [24]. 300 µL protein solution was added to 900 µL substrate mixture (sodium phosphate buffer 0.05 M pH 8: Ethanol: substrate solution 10 mM = 95:4:1). Reaction mixture incubated at 50 °C pH 8 for 15 min. Reaction process stopped by incubating the mixture on ice. One Unit of lipase is defined as the amount of enzyme releasing 1 µmol pNP /min. Specific activity defined as Unit/mg proteins.

To probe substrate specificity assay C10-C16 analogue substrate (p-nitrophenyl Decanoate/ pNPD, p-nitrophenol Laurate/ pNPL, p-nitrophenyl Myristate/ pNPNM and p-nitrophenyl Palmitate/ pNPp) were used for the reaction. Reaction mixture was incubated in standard reaction as described before. To probe optimum temperature, the assay was performed using pNPD as substrate at temperature range at 30–80 °C, pH 8. Reaction was performed in standard reaction described before. To measure optimum pH, the assay was performed using pNPD as substrate and reaction mixture incubated at various pH (sodium phosphate buffer 0.05 M for pH 6–8 and glycine-NaOH buffer for pH 9–11) at 15 min. To monitor lipase activity in the presence of organic solvents, methanol, ethanol, isopropanol, aceton, acetonitrile, chloroform and n-hexane at 3% concentration were used. Assay was performed at 40 °C, pH 9 and use standard reaction as described before. To probe stability of enzyme at 40 °C, enzyme was firstly incubated on that condition for 0–240 min. Following incubation, the assay was performed at 40 °C, pH 9 and use standard reaction as described before.

3. Result and discussion

3.1. Lipolytic activity of _Lk4 H1104_ mutant

Histidine at position 110 of Lk4 was confirmed as one of oxyanion hole [21]. The residue was replaced into phenylalanine through PCR directed mutagenesis. Mutation of _H_ to _F_ was carried out since the amino acids structurally similar, both residues contain bulky side chain, however, histidine contains free electron pair as nucleophile while phenylalanine lack of nucleophily property. The gene was inserted on the expression vector pET30a(+) fused by His-tag. Following expression of the gene, the protein was purified by IMAC NiNTA. Both proteins (wild type and mutant) exhibited maximum lipolytic (ester hydrolysis) activity on
para-nitrophenyl decanolate (pNPD) as substrate. The mutant protein showed higher specific activity (0.0508 U/mg) compared to that wild type (0.0115 U/mg) (Table 1). Increasing the activity of mutant enzyme up to 4 times was not surprising since substitution of residue on oxyanion hole might change conformation of catalytic pocket. Oxyanion hole residue of lipase is conformationally close to catalytic residues. Substitution on the residue might change the interaction of catalytic residues with substrate as shown on mutation at I12 F Bacillus subtilis lipase [16]. In case of Lk4 H110 F mutant, the activity of the enzyme significantly increase, might due to change in local conformation of the enzyme especially on surrounding of the catalytic pocket since phenylalanine carrying large hydrophobic aromatic side group. As consequence, the substitution might change orientation of the NH group on the oxyanion hole so that increasing stabilization of transition state and hence increasing activity of the enzyme [32].

To further probe the molecular mechanism of the above phenomenon, molecular docking assay was subjected to enzymes using pNPD as substrate. The result showed that both enzyme (Lk4 and Lk4 H110 F) exhibited same estimated binding energy with value of ~6.7 kcal/mol. On the other hand, protein–ligand coordinate plot using Ligplot” program showed that hydrogen bond between amino (-NH) residue of oxyanone hole and oxygen (O) residue at C1 of pNPD is shorter in the mutant (3.07 Å) compared to that in the wild type (3.08 Å). In mutant enzyme the substrate was seen closer to viscosity to oxygen (O) residue at gamma position of nucleophilic residue (S109) (Fig. 1). In which serine will form covalent bond with substrate during intermediate formation [33]. Further characterization on the substrate-protein interaction distance using PLIP program revealed that the distance of hydrogen bond involving S109 and salt bridges involving H277 between enzyme and substrate were shorter in mutant compared that the wild type (Table 2). Both data are in agreement that substitution on histidine to phenylalanine at Lk4 might change on local conformation of catalytic pocket and hence increasing activity of the enzyme.

3.2. Substrate preference

Most of lipases are specific to a particular type of substrate especially on carbon length of substrate [34]. A few lipases are also specific to substrate containing double bonds on particular position [15]. Mutation at D94 S on oxyanion hole of Rhizopus oryzae lipase (ROL) caused shifting toward shorter carbon chain substrate [14]. Geometry and hydrophobicity of catalytic pocket determine chain length specificity of lipase. Although geometry at catalytic triad is highly conserve, high variability of the catalytic pocket cause different specificity of lipase for particular substrate [24]. It is also determined by hydrophobic interaction between catalytic pocket residues and ligand’s acyl chain [35]. Mutation of Lk4 at H110 F seem slightly alter specificity of the enzyme on preferencies of the substrates (Fig. 2). Both enzymes showed highest activity on pNPD as substrate. Moreover the wild type exhibited lost of activity on C14 and C16 carbon chain substrate meanwhile the activity of mutant still remained up to 20 %.

Table 1

|       | Total Activity (U) | Specific Activity (U/mg) | Yield (%) | Purification fold (x) |
|-------|--------------------|--------------------------|-----------|-----------------------|
| Lk4   | CE                 | 0.1172                   | 0.0017    | 100                   | 1                      |
|       | P                  | 0.0067                   | 0.0115    | 6                     | 6,9339                 |
| Lk4 H110F | CE              | 0.0954                   | 0.0023    | 100                   | 1                      |
|       | P                  | 0.0563                   | 0.0508    | 59                    | 22,4010                |

Fig. 1. Enzyme-substrate interaction coordinate. A: Lk4-pNPD, B: Lk4H110F-pNPD.
Table 2
Enzyme-substrate interaction distance created using PLIP.

|                  | Lk4 Residu involved | Distance (Å) |
|------------------|---------------------|--------------|
| Hydrogen bond    | S109                | 2.74         |
|                  | H110                | 3.08         |
| Salt bridge      | H277                | 5.1          |

|                  | Lk4 H110 F Residu involved | Distance (Å) |
|------------------|-----------------------------|--------------|
|                  | S109                        | 2.6          |
|                  | F110                        | 3.07         |
|                  | H277                        | 4.96         |

Fig. 2. Activity of Lk4 and Lk4H110 F on various different substrate. The assay was carried out at 50 °C, pH9.

3.3. Effect of organic solvents on enzyme activity

In the presence of organic solvent, most of enzymes are easily denatured and inactivated. However, some lipases still remain functional since the enzymes have rigid conformation and active under two phase systems [36]. Lk4 exhibited some variation activities in polar organic solvent. However, the enzyme was completely lost of the activity in the presence of nonpolar organic solvent such as chloroform or n-hexane (Fig. 3). In contrast, the mutant enzyme still showed 50% activity in the presence of n-hexane compared to that the control (ethanol). Improving activity of the enzyme in the present of organic solvent was reported on F17 S mutant of B. thermocatenolatus lipase [18]. Our previous report showed that lipase from Pseudoxantomonas sp. still exhibited an activity in the presence of methanol up to 30% concentration [37]. Furthermore, lipase from Geobacillus thermo-levorans showed tolerance to various polar solvents and loss of activity in the presence of n-hexane and chloroform [38]. In conclusion, hydrolytic activity of lipases was higher in the presence of water-miscible solvent compared to that on water immiscible solvent. Remaining 50% activity of the mutant (Lk4 H1104) in the presence of n-hexane might be due to substitution of histidine to phenylalanine changed on polarity of oxyanion hole influencing on conformation of catalytic pocket. As consequence the catalytic activity of the enzyme was still retained on the presence of nonpolar organic solvent.

3.4. Effect of temperature and pH on enzyme activity

Lipases from various Pseudomonas species have been studied extensively. Each lipase exhibited optimum activity on diverse temperature: P. fluorescens lipase (40 °C) [39], Pseudomonas sp. f-B-24 lipase (40 °C) [40], P. aeruginosa KM110 lipase (45 °C) [41], Pseudomonas sp. AMS3 lipase (50 °C) [42], P. aeruginosa SRT9 lipase (55 °C) [43]. To probe the effect of the temperature on the activity of enzymes, the mutant and wild type enzymes were assayed at variation temperature from 30 up to 80 °C. Both enzymes showed optimum temperature at 40 °C. The activity of Lk4 showed insignificant difference at temperature range from 40–60 °C (Fig. 4). Surprisingly the activity of mutant (Lk4 H110 F) enzyme was dramatically decreased at temperature above 40 °C. Substitution from histidine to phenylalanine might changed electrostatic force of the residue from negative charge (H) to nonpolar (F) residue and increased steric hindrance on the oxyanion hole. For more detail to slightly evaluate substitution from H to F on steric hindrance effect, total cavities of both enzymes were determined through Voronoi program. The result showed that total cavities of mutant was higher compared to that the wild type (Table 3). It is suggesting that an alleviation temperature in the mutant influences on conformational stability of mutant enzyme and hence destabilization of intermediate enzyme complex.

Most of lipase showed optimum activity at neutral to slight alkaline pH [44,45]. Lk4 showed an alkaline lipase with optimum activity at pH 9 (Fig. 5). Most of Pseudomonas lipases were reported exhibited optimum pH at pH 7–8, only a few of them showed optimum pH at 9 [42]. Optimum pH is determined by some factors such as composition of amino acids (pl), and arrangement of titratable groups in protein structure [46]. Isoelectric point of Lk4 and Lk4 H110 F are 6.13 and 6.05 respectively. Meanwhile both enzymes showed optimum activity at pH 9. It seems that the enzymes should be negatively charge to perform the activity. At pH...
Table 3
Total cavities prediction of wild type and mutant lipases.

| Lipase  | Total Cavities (Voronoi) |
|---------|--------------------------|
| Lk4     | 18                       |
| Lk4H110F| 22                       |

3.5. Thermostability of enzymes

The optimum temperature of Lk4 and Lk4 H110 F were at 40 °C, moreover the activity of Lk4 remained similar up to temperature at 60 °C (Fig. 5). In contrast, the mutant enzyme was lost of the activity up to more than 75 % at 60 °C. For further analysis concerning thermostability of the enzymes, both enzymes were assayed by incubation up to 4 h at the optimum temperature (40 °C) and pH 9 (Fig. 6). After 4 h incubation, the wild type enzyme was shown relatively stable, remaining activity of 97 %. In another hand, after 4 h incubation remained activity of the mutant was 87 %. The higher loss of mutant activity compared to that the wild type was in line with the predicted total cavity of the molecule. Higher total cavity resulted on more water molecules buried in it and caused destabilization of Van der Walls and hydrophobic interaction among amino acid residues [47]. It seems that the role of oxyanion hole residues do not only to bind substrate but also regulate conformational stability of the enzyme. Since oxyanion holes residues lied inside the catalytic pocket, substitution on oxyanion residues might change the protein compactness and hence changes stability of protein [16]. In the case of the mutant enzyme showing higher activity but less stability is probably due to related on structural stability of the mutant. In many cases, enhancing protein flexibility leading on increasing protein activity but less stability [48]. Mutation at F17 S involved on oxyanion formation in chimeric lipase from *B. thermostaurophilus* also show increasing activity but decreasing thermostability [18].

From all of the data suggesting that mutation on H110 F on Lk4 showed changing on substrate-enzyme interaction resulting on increasing activity, shifting toward longer carbon chain substrate, improving the activity in the present of nonpolar organic solvent, however slightly decreasing in thermal stability of the enzyme.

4. Conclusion

Expression vector of pET-Lk4 and pET-Lk4H110 F were successfully constructed and expressed in *E. coli* BL21 (DE3). Crude extract of both proteins exhibited lipolytic activity on pNPD as substrate. The purified enzymes showed same optimum pH and temperature, substrate preference, and maintained the activities in presence of polar or semipolar organic solvent. The activity of mutant enzyme showed much higher catalytic activity compared to that the wild type. In addition, the wild type enzyme lost of its activity in nonpolar organic solvent and long carbon chain (C14 and C16) substrates meanwhile the mutant still exhibited the activity.

Author contribution statement

All authors listed have made a substantial, direct and intellectual contribution to the work and approved it for publication.

Additional information

No additional information is available for this paper.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.j.btre.2021.e00590.

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