Journal of Molecular Catalysis B: Enzymatic 61 (2009) 162–167

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

The effect of substitution of Phe^{181} and Phe^{182} with Ala on activity, substrate specificity and stabilization of substrate at the active site of *Bacillus thermocatenulatus* lipase

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ARTICLE INFO

Article history:
Received 3 May 2009
Accepted 11 June 2009
Available online 18 June 2009

Keywords:
Bacillus thermocatenulatus
Lipase
Lid
Steric hindrance
van der Waals
Site-directed mutagenesis

ABSTRACT

Steric hindrance leads to limitation in the access of substrate into the enzyme active site. In order to decrease steric hindrance, two conserved residues, Phe^{181} and Phe^{182}, in the lid domain of *Bacillus thermocatenulatus* lipase were substituted with alanine by using site-directed mutagenesis. As a result, three mutant lipases were produced. Circular dichroism (CD) spectroscopy showed that the secondary structure of all lipases is similar to one another. F181A mutation increased the distance between phe^{181} and catalytic ser^{114}, which is buried in the active site by 3.24 Å. It can be suggested that such an increase in distance may lead to a decrease in steric hindrance. F181A mutation increased overall lipase activity by up to 2.6-fold (4670 U mg^{-1}) toward C8 substrate. It also resulted in optimal lipase activity at 65 °C rather than 55 °C. F182A mutation increased the distance between phe^{182} and catalytic ser^{114} by 1.54 Å but failed to induce any significant effect on lipase activity. However, F181A–F182A mutation significantly decreased the activity due to decreased van der Waals interactions between the phenyl group of phenylalanines and the acyl chain of triacylglycerol. These results indicate that presence of one of the two residues, Phe^{181} or Phe^{182}, is important for stabilizing triacylglycerols in active site.

1. Introduction

Lipases (E.C. 3.1.1.3) are enzymes that hydrolyze the ester bond between acyl groups and glycerol in triacylglycerides at the oil–water interface, the products of which are glycerol and fatty acids [1]. Lipases are used in various industries, from detergent additives to fine chemical synthesis [2]. *Bacillus thermocatenulatus* lipase (BTL2) has shown highest activity toward tributyrin (C4) as substrate [3] and contains a "lid" (residues 176–189 in BTL2 sequence) covering the substrate binding pocket [4]. For access of the substrates to the active site, displacement of the lid is necessary [5]. Mutations in the lid domain have been the subject of recent studies that have aimed to increase activity and enhance other properties of lipases such as acyl chain length preference and increase in temperature stability [6–8]. In this study we increased lipase activity and substrate specificity of the BTL2 lipase towards longer acyl chains, through a decrease in steric hindrance by substituting bulky side residues with small side chain residue(s). To achieve the above goals, site-directed mutagenesis was used to introduce three different mutations; either single (F181A and F182A) or double mutations (F181A–F182A) into the *btl2* gene. In order to investigate the role of the lid region in substrate stabilization at the active site through van der Waals interactions, focus was placed upon two conserved residues (Phe^{181} and Phe^{182}) of the lid region. The role of steric hindrance of the phenyl group side chain of Phe^{181} in allowing access of substrate to the active site was also evaluated. Interestingly, substitution of two residues with alanine affected not only lipase activity but also substrate specificity of the *Bacillus thermocatenulatus* lipase.

2. Materials and methods

2.1. Bacterial strains, plasmids and cultivation

Bacillus thermocatenulatus (DSM 730) was used for genomic DNA preparation. *Escherichia coli* DH5α and plasmid pTZ57R/T (Fermentas) were used in cloning experiments and *E. coli* BL21 (DE3) and pET-26b (+) (Novagen) were used for the purpose of expression. *B. thermocatenulatus* (DSM 730) was grown as reported previously [9]. *E. coli* DH5α and Bl21 were grown in LB medium at 37 °C.

2.2. Cloning of the btl2 gene and site-directed mutagenesis

Genomic DNA of *B. thermocatenulatus* was isolated according to Sambrook et al. [10]. The *btl2* gene was amplified by PCR based on available sequences (GenBank accession no. X95309) using the

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doi:10.1016/j.molcatb.2009.06.006

Table 1
The primers used in this study.

Name	Sequence	Enzyme site
BTL2.F1	5-GA**TGGCCA**TGGCGGCATCCCCACGCGCC-3	*Mlu NI*
BTL2.R4	5-TT**GAGCTC**ATCATCCCTTCATTAAGGCCGC-3	*Sac I*
F181A.R2	5-GCTGGTCAAAG**GC**GCGATCAG-3	
F181A.F3	5-CTGATCGC**GC**CTTTGACCTGC-3	
F182A.R2	5-GCAGGTCA**GC**GAAGCGATCAG-3	
F182A.F3	5-CTGATCGCTTC**GC**TGACCTGC-3	
F8182A.R2	5-CAGGTCAGCG**GC**GCGATCAG-3	
F8182A.F3	5-CTGATCGC**GC**CGCTGACCTG-3	

primer pair BTL2.F1 and BTL2.R4 (Table 1). The 1198 bp amplicon was cloned into pET-26b(+) leading to the formation of the pYRK^P-BTL2 plasmid which was then introduced into *E. coli* BL21 (DE3). The resulting transformants were selected on LB plates supplemented with kanamycin (30 μg ml^{-1}).

The lipase gene (*btl2*) was mutagenized using splicing by the overlap extension method (SOE) [11] which involved the application of primers illustrated in Table 1 and the pYRK^P-BTL2 plasmid as template. The 562 and 658 bp amplicons from first and second results of PCR were purified and used as a template for the third PCR. The 1198 bp amplicon obtained from the third PCR was cloned into pET-26b(+). Expression plasmids derived from pET-26b(+), which carry different *btl*2 mutant genes, were named pYRK^P-F181A, pYRK^P-F182A and pYRK^P-F181A–F182A. The presence of the mutations was confirmed by DNA sequencing. The plasmids were introduced into *E. coli* BL21 (DE3) and transformants were selected on LB plates supplemented with kanamycin (30 μg ml^{-1}).

2.3. SDS-PAGE and western blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli [12]. The western blot was carried out as previously described in [13,14].

2.4. Expression and purification of recombinant lipases

2.5 ml of an over night culture *E. coli* was inoculated into 250 ml of LB medium supplemented with kanamycin (30 μg ml^{-1}) and incubated at 37 °C with shaking of 220 rpm, until an optical density (OD_{600nm}) of 0.6 was reached. Subsequently, IPTG (0.2 mM) was added to induce protein expression at 37 °C for 2.5 h (220 rpm) with additional shaking (150 rpm) at 30 °C for 16 h. *E. coli* cells were then harvested by centrifugation (2500 rpm, 4 °C, 10 min). The cells were re-suspended in lysis buffer [20 mM Tris–HCl, (pH 8.5) 0.5% (v/v) Triton X-100, 10 mM EDTA, 100 mM NaCl, 1 mM PMSF, 1 mM DTT], supplemented with 10 μg ml^{-1} lysozyme and 100 μg ml^{-1} DNase and then stirred for 30 min at room temperature. The lysate was sonicated 3 times (3× 1 min) at maximum speed and centrifuged (10,000 × *g*, 4 °C, 10 min). The supernatant was separated and the pellet (inclusion bodies) washed three times with washing buffer (20 mM Tris–HCl, 100 mM NaCl, 10 mM EDTA, pH 8.5).

The inclusion bodies were resuspended in solubilization buffer [20 mM Tris–HCl (pH 8.5), 8 M urea, 10 mM DTT] to a final concentration of 5 mg ml^{-1}, stirred for 1 h at room temperature and then centrifuged (10,000 × *g*, 4 °C, 10 min). The supernatant was separated, and diluted drop wise to a final protein concentration of 0.1 mg ml^{-1} with refolding buffer [400 mM L-arginine monohydrochloride, 100 mM Tris–HCl (pH 8.5), 100 mM NaCl, 100 mM glycine, 10 mM DTT, 5% (v/v) glycerol]. The protein solution was dialyzed [20 mM Tris–HCl (pH 8.5)] with three exchanges of buffer. Ultimately, insoluble materials were removed by centrifugation at 10,000 × *g* for 10 min at 4 °C.

The wild type and the mutant lipases were purified using one-step ion-exchange chromatography [13]. The crude lipase solution was dialyzed [20 mM Tris–HCl (pH 6.8)] at 4 °C with three exchanges of buffer. Dialyzed solution was passed through a DE-52 cellulose column (Whatman, Maidstone, England) pre-equilibrated with dialysis buffer, and the unbounded protein was immediately collected as purified lipase. The purified lipases were dialyzed [10 mM Tris–HCl (pH 8.5)] at 4 °C with three exchanges of buffer and kept at −25 °C for the next step.

2.5. Circular dichroism

Circular dichroism (CD) spectra in the Far-UV region (190–260 nm) were obtained on an Aviv 215 spectropolarimeter (Aviv Associates, Lakewood, NJ, USA), using a 0.5 mm path cell. CD experiments were carried out using 0.2 mg ml^{-1} of wild type and mutant lipases in 50 mM sodium acetate buffer (pH 8.0) at 25 °C. Five scans were collected for each sample using a step size of 1.0 nm and averaging time of 1 s.

2.6. Lipase assay

Lipase activity was determined with triacylglycerol substrates in a pH-stat (Metrohm Ltd., Herisau, Switzerland). 2 mg ml^{-1} of tributyrin (C4), tricaproin (C6), tricaprylin (C8), tricaprin (C10), trilaurin (C12), trimyristin (C14), tripalmitin (C16), and olive oil (C18) (Sigma) were emulsified in distilled water containing arabic gum (20 mg ml^{-1}) using ultrasonic treatment (Hielscher GmbH, Teltow, Germany) for 5 min at maximum power [9]. 20 ml of substrate solution was adjusted to pH 8.5 at 55 °C, subsequently purified recombinant lipase was added to the substrate solution and the liberated fatty acids were titrated automatically with 0.05 M NaOH. Lipase activity was calculated by the amount of NaOH needed to maintain the pH at 8.5. One unit was defined as the amount of enzyme that released 1.0 μmol of fatty acid per minute [3].

2.7. Effect of temperature, metal ions, organic solvents and detergents on lipase activity

The effect of temperature on lipase activity was studied by carrying out the enzyme reaction at different temperatures in the range of 40–70 °C in a pH-stat by using tricaprylin (C8) as substrate (pH 8.5).

The effect of detergents, organic solvents and metal ions on lipase activity was determined by incubating the enzyme with detergents (1%, w/v) for 2 h at 30 °C, pH 8.5. In the case of organic solvents (30%) and metal ions (1%, w/v), incubation was for 3 h at 30 °C, pH 8.5. Subsequently residual activity of lipase was determined by pH-stat at 55 °C, pH 8.5 by using tricaprylin (in the case of detergents) and trilaurin (in the case of organic solvents and metal ions) as substrates.

3. Results and discussion

3.1. Site-directed mutagenesis

In order to improve activity and substrate specificity of *B. thermocatenulatus* lipase, rational protein design was applied to modify the lid domain. Selection of amino acid residue for substitution was done based on three-dimensional structure of *B. stearothermophilus* P1 lipase (PDB ID, 1JI3) with which it shares 95% sequence similarity with the *B. thermocatenulatus* lipase [4]. By analyzing the crystal structure of *B. stearothermophilus* P1 lipase, it was found that the bulky and highly hydrophobic side chains of the two phenylalanine residues, Phe^{181} and Phe^{182}, of the lid

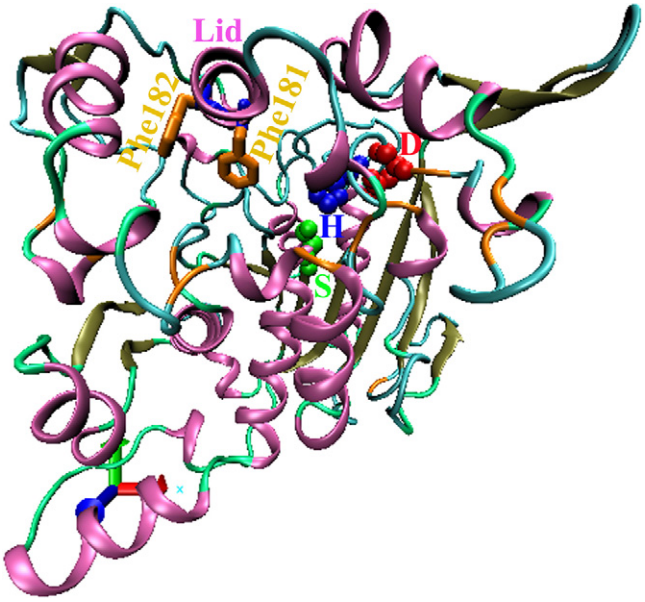

Fig. 1. The Newribbons representation of three-dimensional structure of the wild type *B. thermocatenulatus lipase*. The Phe[181] and Phe[182] residues are shown in orange and the residues of the catalytic triad (Ser[114], His[359], Asp[318]) are shown in green, blue and red colors, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

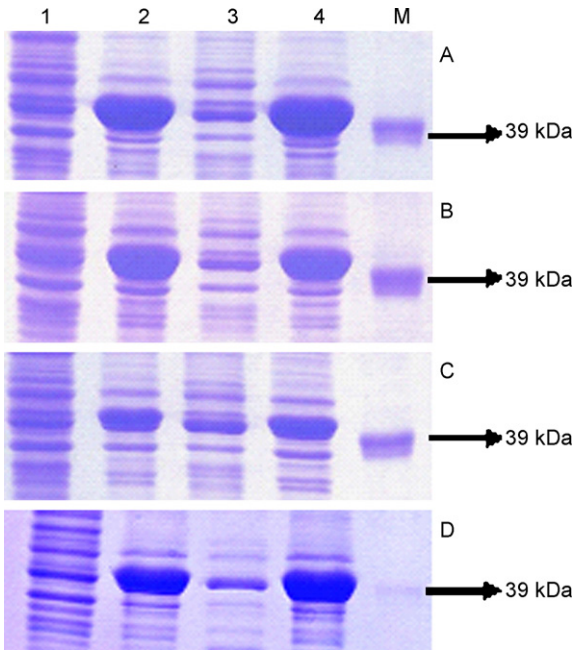

Fig. 2. SDS-PAGE analysis of the crude cell extract and osmotic shock preparation of recombinant clones. Crude extract of *E. coli* expressing either (A) wild type, (B) F181A, (C) F182A and (D) F181-182A recombinant lipases. Lanes 1 and 2, Crude cell extract of recombinant clones before and after induction; Lanes 3 and 4, soluble and insoluble fraction of the osmotic shock preparation; Lane M, molecular weight marker (SM 0431, Fermentas).

domain are oriented toward the active site. It was suggested that these bulky side chains might impede accessibility of substrate to the active site due to steric hindrance. To achieve the above objectives, phe[181] and phe[182] residues were substituted with alanine either by single or double mutations in the *btl2* gene and thus three mutant lipases were constructed (F181A, F182A, F181A–F182A). In addition, the tertiary structure of the recombinant wild type (Fig. 1) and mutant lipases were predicted based on the structure of the *B. stearothermophilus* P1 lipase, using the automated homology modeling program (ESyPred3D web server 1.0, www.fundp.ac.be/sciences/biologie/urbm/bioinfo/esypred/). The results were visualized using the VMD 1.8.6 software [15]. Superimposition of the recombinant wild type and mutant lipases showed no structural change (data not shown).

3.2. Protein expression and purification

Following site-directed mutagenesis, the wild type and mutant *btl2* genes were sub-cloned down stream of the *Pel*B signal sequence under the control of the T7 promoter of pET-26b(+) and were expressed in *E. coli* BL21 (DE3). SDS-PAGE analysis of the crude cell extract showed a major polypeptide band with an approximate molecular weight of 43 kDa (Fig. 2). By applying osmotic shock, it was found that lipases were in the insoluble fraction. Hence, lipase inclusion bodies were isolated after cell breakage, solubilized with 8 M urea and refolded in refolding buffer. Finally, refolded lipases were purified using the one-step ion exchange chromatography method. Subsequently the purity of lipases were evaluated by the western blot procedure (Fig. 3). Arie [16] have also reported inclusion body formation in the periplasmic space of *E. coli* due to the presence of hydrophobic sequences in the expressed recombinant protein. In addition, the presence of hydrophobic patches in lipase increases the tendency of lipase to form insoluble aggregates [17,18]. Therefore, it can be concluded that formation of lipase aggregates in the periplasmic space of *E. coli* is a result of misfolding and intermolecular interactions between hydrophobic patches of the lipase.

3.3. Analysis of secondary structure

The effect of mutations on the structure of lipase was studied by using bioinformatics based on the three-dimensional structure. Superimposing residues corresponding to the lid domain ([176]DFTDRFFDLQKAVL[189]) of the three variants onto the wild type the identical positions and were not altered as a result of mutations (Fig. 4).

To verify whether mutations affected the secondary structure of lipase, wild type and mutant lipases were subjected to CD spectroscopy. Results indicated that the Far-UV CD spectra of all lipases were highly similar to one another (Fig. 5), suggesting that the overall secondary structures of mutant lipases were not altered by mutations.

3.4. Lipase activity and substrate specificity

Activities of the wild type and mutant lipases were assayed in a pH-stat (55 °C in pH 8.5) with the triacylglyceride substrates (C4–C18). Data shows that F181A mutations changed substrate

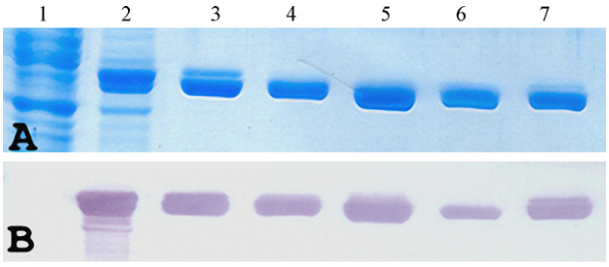

Fig. 3. SDS-PAGE analysis (A) and western blot (B) of the purified recombinant lipases. Protein lanes in panel (A) correspond to the same in panel (B). Lanes 1 and 2, Crude cell extract of *E. coli Bl*21 (DE3) harboring recombinant plasmids before and after induction; Lanes 3 and 4, purified recombinant wild type lipase; Lanes 5–7, purified F181A, F182A and F181A–F182A lipases, respectively.

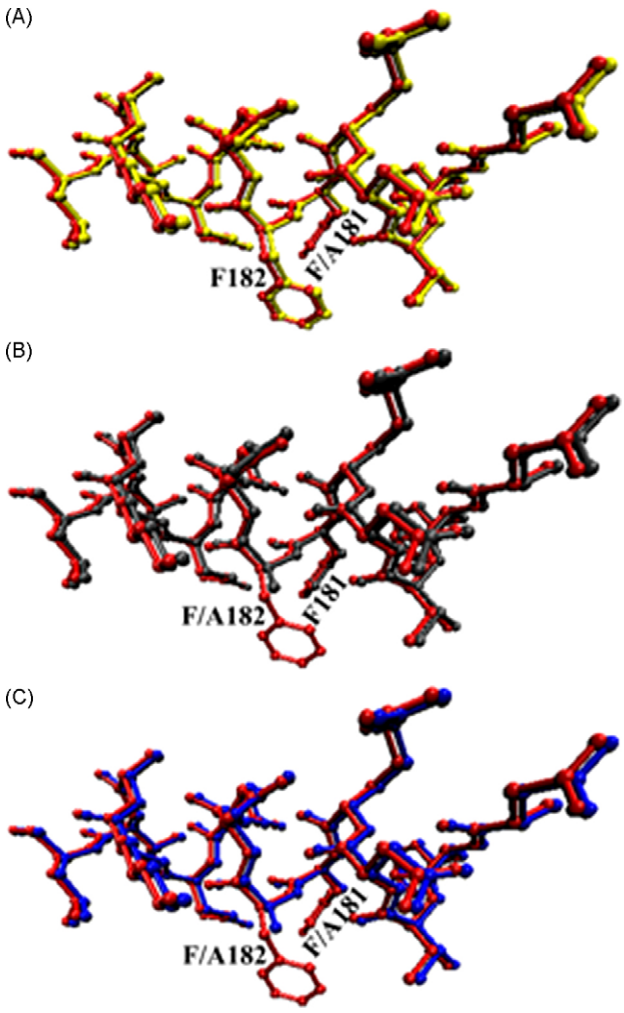

Fig. 4. Superimposition diagram of the lid domain residues of mutant lipases (A) F181A, (B) F182A and (C) F181A–F182A onto the recombinant wild type lipase, respectively. The lids of wild type, F181A, F182A and F181A–F182A lipases are shown in red, yellow, black and blue colors, respectively. All structures were presented with VMD 1.8.6. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

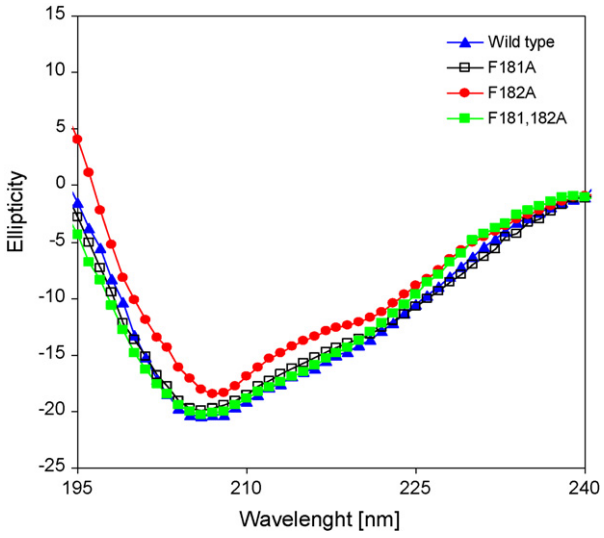

Fig. 5. Far-UV CD spectra of recombinant wild type and mutant lipases. The spectra were measured in 50 mM sodium acetate buffer (pH 8.0) at 25 °C.

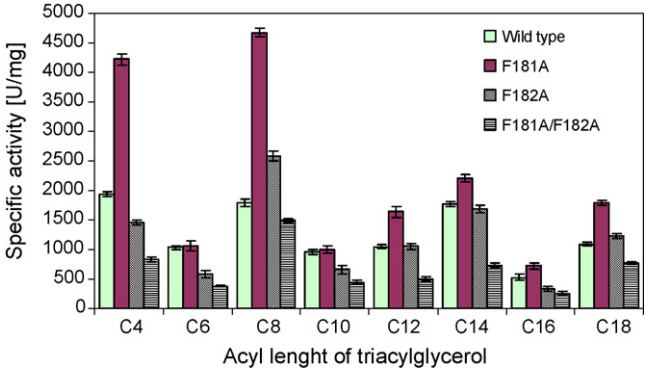

Fig. 6. Activities and substrate specificities of the wild type lipase and three mutant lipases; F181A, F182A and F181A–F182A in the presence of triacylglyceride with different acyl chain lengths as substrates. Activity was measured at 55 °C and pH 8.5.

specificity toward tricaprylin (C8) by demonstrating an increase of approximately 2.6-fold in activity when compared to that of the wild type lipase (4670 U mg^{-1}). In addition, compared with the wild type lipase, F181A mutation increased lipase activity for the entire range of triacylglycerides, such that increases of; 2.2-fold with C4, 1.1-fold with C6, 2.6-fold with C8, 1.1-fold with C10, 1.6-fold with C12, 1.2-fold with C14, 1.4-fold with C16 and 1.6-fold in presence of C18 as substrates were observed (Fig. 6). In contrast, the F182A mutation only changed substrate specificity toward tricaprylin (C8) by an increase to approximately 1.5-fold in activity when compared to that of the wild type lipase (2585 U mg^{-1}), whereas it failed to demonstrate any significant effect on lipase activity. On the other hand the double mutation (F181A–F182A) slightly changed substrate specificity toward tricaprylin (C8) but effectively decreased the general lipase activity (Fig. 6).

In the wild type lipase, due to the bulky side chain of Phe^{181}, the generated steric hindrance may restrict access of substrate into the active site. Since the phenyl side chain of this residue is considerably bulkier than the methyl side chain of alanine, it might restrict access of the substrate into the active site. It could be concluded that Phe^{181} is located along the entry path of the substrate into the active site. These observations are in accordance with a former report and explain why the maximum activity and specificity of *B. thermocatenulatus* lipase is toward small triglyceride substrates such as tributyrin (C4) [3]. The catalytic triad at the lipase active site includes Ser^{114}, His^{359} and Asp^{318}. The distance between the phenyl side chain of Phe^{181} and hydroxyl group of Ser^{114} in recombinant wild type lipase was measured and found to be 7.97 Å. In contrast, this distance might be increased to 11.21 Å following substitution of Phe^{181} with alanine (Phe/Ala^{181}). As a result, increasing the distance by 3.24 Å could increase lipase activity of lipase due to a decreased in steric hindrance and thus enable access of substrates into the active site.

Phe^{182} is a conserved residue in the lid domain which might be involved in the stabilization of substrate at the active site. Homology model showed that the side chains of the Phe^{181} and Phe^{182} residues point to different directions which might explain the behavior of mutated lipase (Fig. 1). Following substitution of Phe^{182} with Ala, the distance between the Ser^{114} and Phe/Ala^{182} side chains increased, however the increased distance was less than that of the F181A mutant and was estimated to be 1.54 Å. While the distance had slightly increased, the enzyme activity was nevertheless seen to decrease. These findings indicate that the role of Phe^{182} is less significant in steric hindrance. Instead it might play a role in stabilization of substrate at the active site through van der Waals interaction with acyl chain of the substrate.

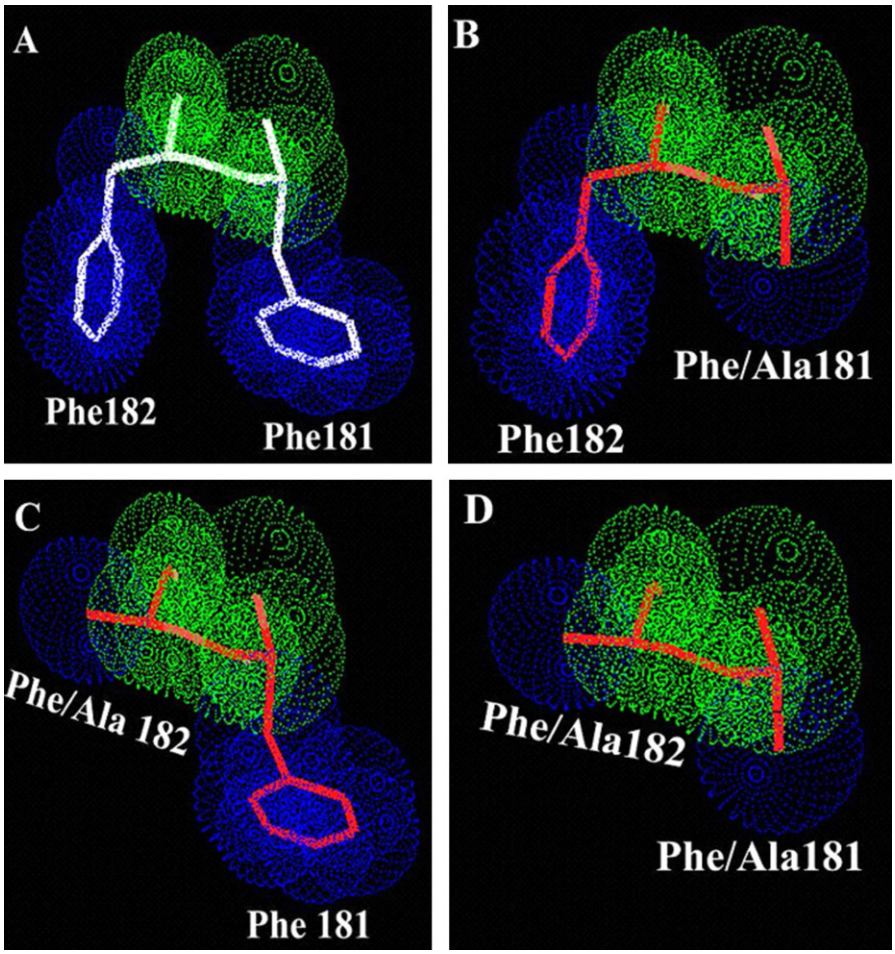

Fig. 7. Schematic representation of van der Waals volume around the side chain of the two conserved residues (Phe^{181} and Phe^{182}) before and after substitution with Ala. (A) Wild type, (B) F181A mutant, (C) F182A mutant and (D) F181A–F182A mutant lipases. In the double mutant lipase, the van der Waals volume around the two conserved residue (Phe^{181} and Phe^{182}) decreased noticeably. Structures were presented with VMD 1.8.6.

As a result of double mutations (F181A–F182A), the volume of the active site as well as the distance between Ser114 and the two mutated residual side chains (Phe/Ala181 and Phe/Ala^{182}) increased by 3.24 and 1.21 Å, respectively, but it also caused an effective decrease in lipase activity. van der Waals interactions are the main forces that keep acyl chains of triacylglycerol at the active site [19,20]. As Fig. 7 shows, decrease in van der Waals volume could take place subsequent to the double mutation (F181A–F182A) (Fig. 7D). When these two residues are replaced with Ala, the van der Waals interactions between the phenyl side chains of these Phes and the acyl chains of triacylglycerol which are important in substrate stabilization at the active site will be decreased. In contrast to single mutations (F181A or F182A), the van der Waals volume around one of the phenyl groups of the Phe side chain remains intact and provides interaction required for stabilization of triacylglycerol at the active site (Fig. 7B and C).

3.5. *Effect of temperatures, metal ions, EDTA, organic solvents and detergents on lipase activity*

The BTL2 lipase has previously been characterized [3,18]. In this study, the effect of mutations on lipase activity in the presence of different temperatures, metal ions, EDTA, organic solvents and detergents was evaluated (Table 2). Results showed that the F181A mutation had lead to optimal lipase activity at 65 °C rather than 55 °C in the case of the wild type lipase. The F181A mutation had lead to increased lipolytic activities in the range of applied temperatures (40–70 °C) in comparison with the wild type lipase (Table 2). Although F182A mutation had increased lipase activities throughout the entire range of applied temperatures, but the increased activity was much less than that of the F181A mutation. On the other hand the double mutation (F181A–F182A) had effectively decreased activity at the entire range of temperatures (Table 2).

The residual activities of lipases following incubation at 30 °C for 3 h with metal ions and EDTA (1%, w/v) were determined. Results showed that when all conditions the F181A mutation increased activity in comparison with the wild type lipase (Table 2). In contrast, EDTA, Ca^{2+} and Fe^{3+} decreased the activity of lipase when compared with that of the control (Table 2). Inhibition of lipase activity by Fe^{3+} has also been reported by Higaki [21]. Similar results have also been reported by Rahman et al. [22] which confirm the results of this study indicating that metal ions such as Ca^{2+} stimulate lipase activity of the *Pseudomonas* sp. strain S5 after 15 min of incubation, whereas prolonged incubation (30 min) leads to decreased enzyme activity. In contrast, the F182A mutation showed almost the same lipase activity as the recombinant wild type lipase. On the other, hand the F181A–F182A lipase exhibited a decreased activity in comparison with the wild type recombinant lipase (Table 2).

In this study, the effects of organic solvents (30%, v/v) and detergents (1%, w/v) on the enzyme activity of the recombinant wild type and mutant lipases were also investigated. Results indicated that in comparison with recombinant wild type lipase, the F181A mutation increased lipase activity in presence of organic solvents (acetone, methanol and 2-propanol) and detergents (Triton-X100, Na-DOC

Table 2
Effect of temperatures, metal ions, EDTA, organic solvents and detergents on lipase activity. Specific activity was determined in a pH-stat (55 °C in pH 8.5).

	F181A–F182A (U ml^{-1})	F182A (U ml^{-1})	F181A (U ml^{-1})	Wild type (U ml^{-1})
Temperatures (°C)[a]				
480	1040	1600	740	40
860	1620	3140	1320	45
900	2120	3860	1560	50
1220	2460	3520	2360	55
740	2420	4260	1480	60
800	2520	4900	1700	65
580	2320	3480	680	70
Metal ions[a]				
1180	2080	4060	2220	Control
1060	1920	4180	1960	Na^+
940	1720	3180	2160	EDTA
1100	2580	4160	2080	K^+
400	420	1920	700	Ca^{2+}
540	380	1980	880	Fe^{3+}
Organic solvents[b]				
680	2020	3880	1980	Control
980	1440	4420	1120	Acetone
520	740	3780	1000	Methanol
700	580	2860	800	2-propanol
Detergents[b]				
680	2020	3880	1980	Control
1400	2380	5220	2520	Triton X-100
1620	2060	4860	2120	Na-DOC
1040	1440	3280	1120	Tween 80
300	300	460	300	SDS

[a] Tricaprylin (C8) was used as substrate.
[b] Trilaurin (C12) was used as substrate.

and Tween 80). Meanwhile, F182A and F181A–F182A mutations conferred decreased lipase activities (Table 2).

Finally, the above data clearly confirmed results that the F181A increased activity, F182A had no significant effect on activity and double mutation dramatically decreased activity. The decrease in lipase activity following a double mutation (F181A–F182A) could be the result of significant reductions in van der Waals interactions between phenylalanine site chain and the acyl chain of the substrate. As a result, the substrate can not be stabilized into the active site which leads to decreased activity. Also, the increase in activity subsequent of F181A mutation result of decrease in steric hindrance, thus allowing easy access of substrate into the active site.

4. Conclusions

Lipases are placed after proteases and carbohydrases in the world regarding the significance of their applicability. These enzymes play important roles in biotechnology and have been used in different industries. In this study we increased lipase activity using rational protein design through a decrease in steric hindrance and easy access of the substrate into the active site. Our results indicate that among three different mutations (F181A, F182A and F181/182A) in the lid domain of the BTL2 lipase, the F181A mutation significantly improves lipase activity under different conditions while the other two mutations not only have no significant effect on enzyme activity but may also decrease enzyme activity. These findings indicate the important role of phenyl groups of these two amino acids in lipase activity, which is either by stabilization of the substrate at the active site through van der Waals interactions or decrease in enzyme activity through steric hindrance. The F181A mutation improves lipase activity due to decreased in steric hindrance. On the other hand the slight decease in lipase activity subsequent to F182A mutation might be due to the fact that stabilization of substrate at the active site is perturbed. In accordance, the significant decrease in lipase activity following double mutations could result from the change in van der Waals volume and subsequent instability of substrate at the active site.

The present report not only shows the role of amino acids with large side chains in the chemistry of lipase but also the importance of applying genetic engineering as well as protein engineering to commercially important enzymes.

Acknowledgements

We would like to thank H. Danesh, Dr. D. Morshedi and Dr. M. Daliri for technical supports. We would also like to thank Dr. M. khodabandeh and Dr. S.A. Ghoreshi for preparation of some materials used in this project and special thanks to Dr. P. Shareati for English editing of this manuscript.

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