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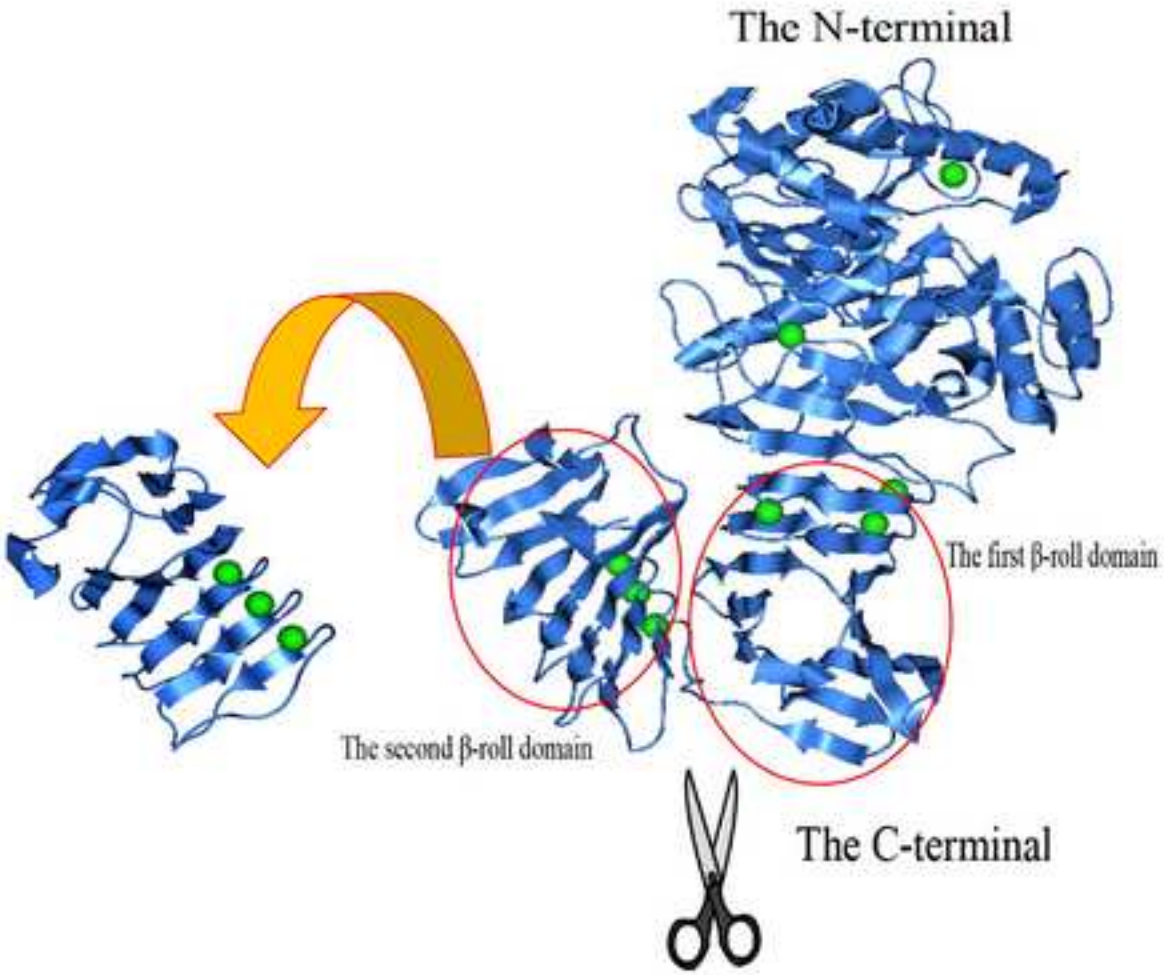
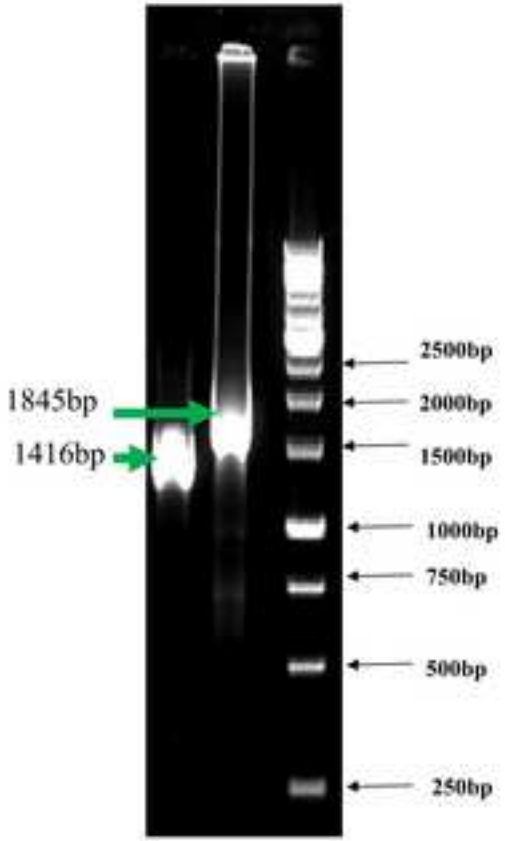
*Graphical Abstract (for review)





Highlights

1- Both wild- type and truncated forms, coding for *lip*A from *Serratia marcescen* were successfully cloned and expressed.
2-Deletion of in the second β-roll domain at the C-terminal region of SML does not deleteriously affect enzyme catalysis.
3- The truncated form of SML with strong catalytic efficiency could be employed potential to industrial applications.

Bacterial expression and characterization of an active recombinant lipase A from *Serratia marcescens* with truncated C-terminal region

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Abstract:

The lipase of *Serratia marcescens* (SML) with 614 amino acid residues belongs to the lipase family I.3 has an important pharmaceutical application in production of chiral precursors. Like other members of this family, the SML consists of the N-catalytic domain (α/β) that contains the active-site residues and the C-terminal domain including the two parallel β-roll domains, the first and second β-roll. The repetitive sequences, a nine-residue sequence motif (GGXGXDXUX), in SML are somewhat degenerated as well as are not consecutive. The parallel β-roll domains separated by a 72 residues spacer from each other. The importance of these repetitive sequences is not yet fully understood. In the present investigation, as an approach, a C-terminally truncated (~13kD) SML was generated using PCR-based site-directed mutagenesis method (designated SML-Δ128). Both wild-type and truncated forms of SML were constructed , overexpressed in *E. coli* without Lip-system and purified by affinity chromatography on the Ni-NTA system. Kinetic parameters and circular dichroism (CD) spectra were determined and compared. The SML-Δ128 showed an approximately 3.7-fold increase in the turnover rate (k_{cat}), relative to that of the full-length enzyme. In conclusion, this report demonstrates that the SML could tolerate extensive modification in the C-terminal extreme of the protein, as deletion of the C-terminal region (~13 kDa), consisting of several tandem repeats of glycine-rich and 49 residues including signal peptide, significantly increases the catalytic efficiency of the enzyme.

Keywords: *Serratia marcescens,* Family I.3, lipase, β-roll, Expression, CD spectra

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1. Introduction

Lipases (triacylglycerol acylhydrolases , E.C. 3.1.1.3), are ubiquitous enzymes that catalyze a wide range of reactions, including hydrolysis, inter-esterification, alcholysis, acidolysis, esterification and aminolysis, hence they are widely used in a variety of industrial applications such as the synthesis of chemicals, pharmaceuticals, etc [1, 2]. Lipases produced widely in nature by various animals, plants and microorganisms, but only lipases of microbial origin, mainly bacterial and fungal, are commercially significant due to their diversity in catalytic activity, high yield and low cost production, as well as the relative ease of genetic manipulation. Moreover, microbial lipases not only are stable in organic solvents but also do not require cofactors, as well are encompass broad substrate specificity [3, 4]. With recent advances in recombinant DNA and protein engineering, various strategies for further enhanced catalytic potential of lipases have been developed [5]. Genetic manipulation to increase the enzyme activity and stability as well as producing new altered enzymes with special features has been employed easily using microbial cells [3, 6]. Bacterial lipases based on the difference in amino acid sequence and biochemical properties have been 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 Gram negative bacteria true lipases [7, 8]. Subfamily I.1 and I.2 lipases share relatively high amino-acid sequence similarity, in-contrast, subfamily I.3 lipases have been shown that possess low amino-acid sequence similarity (<20%) to either family I.1 or family I.2 lipases [9]. In addition, those have higher molecular size than lipases from subfamilies I.1 and I.2 and both lack of Cys residue [10]. The lipases from *Serratia marcescens* (SML) and *Pseudomonas fluorescens* belonging to the subfamily I.3 are separated from other lipases not only by their amino acid sequences, but also by their secretion mechanisms and biological properties [8, 11]. *S. marcescens* lipase has 56-66% homology with the lipases of *Pseudomonas fluorescens* that both have no conventional N-terminal signal sequence, but contain a C-terminal sequence consisting of multiple repeats of nine amino acid residues which has been found in others extracellular proteins of gram-negative bacteria that are secreted via the type I secretion system (T1SS) such as metalloprotease from *Erwinia chrysanthemi* ,Hemolysin in *Escherichia coli* , Leukotoxin in *Pasteurella haemolytica* , cyclolysin in *Bordetella pertussis* and Ca^{+2}-binding protein in *Rhizobium leguminosarum* [12, 13]. SML consists of 613-614 amino acid residues with molecular weight of about 64-65kDa and two domains. Likewise the N-catalytic domain contains the active site residues, Ser^{207}, Asp^{256}, and His^{314} (catalytic triad) where the serine residue is located into a GXSXG motif that forms a sharp γ-like turn between a β-strand and the following α-helix [14, 15]. The C-terminal domain , like other family I.3 lipase, contains several tandem repeats of glycine-rich nine-residue sequence motif $(GGxGxDxux)_n$ (u: hydrophobic amino acid residue, x: any amino acid residue) , in the upstream region of the C-terminal , and a putative secretion signal near the C-terminus which are so-called RTX (repeats in toxin) family proteins [14, 16]. The lipase A from *Serratia marcescenss* 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 [14]. According to the crystal structures of metalloprotease from *S. marcescens*, repetitive sequences form parallele β-roll motif, that several Ca^{2+} ions bind to the first six residues of this sequence motif to form a loop and the remaining three residues form a β-strand that each Ca^{2+} ion binds between a pair of these loops [17]. The crystal structure of SML in an open conformation has been determined which resembles a family I.3 lipase from *Pseudomonas* sp. MIS38 (PML) [9, 14]. According to these structure , the N-catalytic domain and the C-terminal β-roll sandwich domain, are nearly identical with each other, except for the structures of two lids (lid 1 and lid 2) and Ca^{2+}-binding sites [15, 18]. Among various family I.3 lipases, a family I.3 lipase from *Pseudomonas* sp. MIS38 (PML) has been structurally and functionally well studied ,the number of repetitive sequences in PML is proposed 12-13 repeats (residues 373-558) , but SML possesses 12-14 repeats in two groups spanning residues, 369-418 and 489-564, and separated by a 70-residue spacer [9, 14, 16, 18]. The role(s) and importance of these repetitive sequences is still debatable and is not fully understood. Analyses of different mutant proteins as partial or complete knock-out of the repetitive sequences, have shown the strongly reduced protein secretion levels [19]. Furthermore, it has been suggested that the repetitive sequences

are not required for secretion but are needed for activity [20] since knock-out of all repeats in PML, a family I.3 lipase, leads to reduced enzymatic activity [21]. Despite this evidence, the results of other studies suggested that point or deletion mutations of each of these repetitive sequences had no effects on the activity and stability of the enzyme [22, 23]. Further studies in this field performed on similar proteins, while no reports have been proposed for SML to show the effect of removing these repetitive sequences on conformation, activity and stability of the enzyme. PCR mutagenesis has been a useful and successful method to identify protein important regions. In the present study, we demonstrated the functional significance of a part of the C-terminal region in the native full-length of SML for enzymatic activity by constructing a C-terminal truncated (~13kD) SML as the SML-Δ128. In order to locate this segment, a structural model of SML was used. The SML-Δ128 was constructed by deleting the eight repetitive sequences and 49 residues including signal peptide (Fig. 1). Based on the results, we have discussed the effects of partial deletion of the C-terminal region of the recombinant lipase from *S. marcescens* (rSML) on its structure and function, compared to the recombinant full-length SML as the "wild-type".

2. Materials and Methods

2.1. Bacterial Strains, Plasmids, and Culture conditions

Marine *Serratia marcescens* , (collection no. UTMS 2342) which is deposited in the Microorganisms Collection of the Microbial Technology and Products (MTP) Research Center of Iran , was utilized as the lipase A gene source for the cloning and expression experiments. *E coli XL1* -Blue [recA1 *endA1 gyrA96 thi-1 hsdR17 supE44 [F' proAB lacI*^{q}*ZΔ15 Tn10 (Tet*^{r}*)*] (Novagen) and *E. coli BL21* (DE3) [*F*$^{-}$ *ompT hsdSB (rB*$^{-}$ *mB* $^{-}$*) gal dcm (lcIts857 ind1 Sam7 nin5 lacUV5-T7 gene1*] (Novagen) were employed as host strains for the gene manipulation and protein expression, respectively. The plasmids used for the cloning and protein expression were pUC19 [*ColE1 Amp*^{r} *lacI φ80dlacZ*] (Stratagene) and pET-28a (+) Kan^{r} (Novagen), respectively. *S. marcescens* was grown in a nutrient broth (0.3% beef extract, 0.5% peptone and 0.5% NaCl) at 30 ◦C. *E.coli* was cultured in a LB broth (1% peptone, 0.5% yeast extract, 1% NaCl) at 37 ◦C, and an appropriate amount of ampicillin (100μg/ml) or Kanamycin (50μg/ml) was added to LB broth for recombinant *E.coli* when needed. Isopropyl β-D-1-thiogalactopyranoside (IPTG) and X-gal were added to the culture media or solid plates when needed. The cell growth was determined by measuring the optical density at 600 nm using a spectrophotometer.

2.2. Protein modeling

A protein model was made for SML (designated SMLΔ128), based on the crystal structure reported of *S. marcescens* lipase (PDB code: 2QUA) [14] with the use of the homology model module of MOE 2012.10 (Molecular Operating Environment; Chemical Computing Group Inc., Montreal, Canada) [24] .The model was used to identify the location of β-roll segments of the enzyme C-terminal region.

2.3. Construction of plasmids for expression of wild-type and truncated SML

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′) for SML-WT. Likewise the gene encoding SML-Δ128 was amplified by PCR using primers 1 and primer 3

(5′- TAGGATCC**TTA**CGCATAGGCTTTGAGGC-3′) where, the underlined bases represent the *Nde*I site for primer 1, and *Bam*HI site for primers 2 and 3. The ATG codon for the initiation of the translation and the sequence complementary, TAA, to the termination codon are shown in bold type. Truncated form of SML (designated SMLΔ128), was created using PCR-based site-directed mutagenesis by introducing a stop codon at amino acid residue 487 of the SML full length gene. The PCR products were digested with with *Nde*I and *Bam*HI , purified, and ligated into the *Nde*I and *Bam*HI sites of pUCl9 vector and was then transformed into XL-1 Blue cells. Plasmids pET-SML and pET- SMLΔ128 for overexpression of SML-WT and SMLΔ128, respectively, were constructed by digesting the plasmid pUCl9 derivatives with *Nde*I and *Bam*HI, followed by ligation of the resultant *Nde*I and *Bam*HI fragments into the *Nde*I and *Bam*HI sites of pET-28(+).The nucleotide sequences of the genes were confirmed by DNA sequencing.

2.3. Overexpression and purification

E.coli BL (DE3) cells were transformed with pET-SML and pET- SMLΔ128 and grown in 200 ml LB broth containing 50μg/ml kanamycin at 37^{o} C. When the absorbance at 600 nm of the culture reached spanning 0.7-0.8, isopropyl-L-D-thiogalactopyranoside (IPTG) with final concentration 1 mM, was added to the culture medium and cultivation was continued for an additional 12 h at 35^{o} C. Cells were harvested by centrifugation at (5000×g , 10 min, 4 ^{o} C) and the pellet was washed twice with 50mM Tris-HCl, pH 8.0 solution. The cell pellet was then re-suspended in buffer A (6 M Guanidine Hydrochloride, 20mM NaH2PO4, 500 mM NaCl, pH 7.8) and 40μg/ml PMSF and the cell lysed disrupted by gentel sonication (6 cycles, 10 s) on ice. Cell debris was then centrifuged (15000 × g, 20 min, 4 ^{o} C) to separate the cells and supernatant. The eight ml of the supernatant fraction containing soluble recombinant lipase was mixed with 1.5 ml of nickel affinity resin (Ni-NTA, Invitrogen) previously equilibrated with buffer B (8 M Urea , 20 mM Sodium Phosphate pH 7.8 , 500 mM NaCl), incubated for 1 h at 22 ^{o} C and applied to an empty column. The column was washed several times with buffer B (pH 6). Finally, recombinant proteins were refolded and eluted with buffer C containing 100 mM Tris- HCl, pH 8.0, 250 mM NaCl, 20 mM $CaCl_2$, and 250 mM imidazole. The fractions containing protein solution were pooled and several times was dialyzed overnight at 4 °C against 50 mM Tris- HCl pH 8.0 buffer containing 150 mM NaCl , 10mM $CaCl_2$. The protein eluted from the column was used for biochemical characterization

2.4. Protein measurement

The protein concentration was determined according to [25]using bovine serum albumin (BSA) as a standard.

2.5.SDS-PAGE

The expression level of rSML (wild- type and truncated lipase) in the cells , molecular mass and the purity of the proteins were analyzed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to [26] followed by staining with Coomassie brilliant blue R-250 and destined with methanol/acetic-acid/water (3:1:6 v/v/v).

2.6. Determination of lipase activity

The lipase activity was determined by a spectrophotometric method at 410nm using *p*-nitrophenyl laurate (*p*-NPC_{12}) as the substrate. The reaction was carried out as follows: **Solution A**: (one part) *p*-nitrophenyl laurate (in isopropanol *)* 5mM (to a final concentration (0.5mM) as substrate , **Solution B**: (nine part) 50mM Tris-HCl (pH 8.0) buffer , containing 5mM $CaCl_2$, 0.1% (w/v) gum arabic 0.2% (w/v) sodium deoxycholate 0.4% (v/v) triton X-100 . Therefore, 950 µl of the substrate solution was mixed with 50 µl of the enzyme solution (20-60 µg) to make a final volume 1ml. The activity assay was done at 42^{o}C, for 5 min. The molar absorptivity of p-nitrophenol in same buffer was experimentally determined as 14200 M^{-1} cm^{-1}. One unit of enzymatic activity was defined as the amount of enzyme that liberated 1 µmol of p-nitrophenol per min under assay condition. The specific activity was defined as the enzymatic activity per mg of protein. The experiments were performed triplet and the processed data were given as the means ±SD of triplicate measurements from three independent determinations for each enzyme.

2.7. Determination of optimum pH and temperature

The effects of temperature and pH on activity were determined spectrophotometrically using *p*-nitrophenyl nitrophenyl laurate (*p*-NPC_{12}) as the substrate. The optimum temperature for enzyme activity was determined by measuring the rate of reaction a temperatures ranging from 30 to 50 ° C. The optimal pH for lipase activity was measured by incubating the enzyme substrate mixture at various pHs (50mM acetate/Na-acetate buffer, (pH 4.0-6-0), 50mM Tris-HCl buffer, (pH 7-9.0) and 50mM glycine/NaOH buffer, (pH 10-12.0). Reactions were carried out by mixing the appropriate buffer with a stock solution of 5 mM *p*NPC_{12} in order to obtain a substrate final concentration of 0.5 mM. The assay reaction was performed similar measurement method that described previously and relative activities (%) were calculated by regarding maximum activity.

2.8. Determination of substrate specificity

The substrate specificity was determined spectrophotometrically using *p*-nitrophenyl esters (Sigma) (*p*NP-acetate (C2), *p*NP-butyrate (C4), *p*NP-laurate (C12), *p*NP-palmitate (C16) as substrates.

2.9. Determination of kinetic parameters and statistical analysis

The kinetics of the enzymes was measured using a spectrophotometric activity assay with varied concentrations of *p*NPC_{12} (0.05–2.0 mM) as substrate of the lipase. The Michaelis–Menten kinetic parameters, of both enzymes were calculated from the experimental data using Sigma plot 12 software. The parameter Catalytic efficiency was also obtained by using the equation *k*cat/*K*m. The data reported in all figures and tables are an average of triplicate observations and were subjected to one-way analysis of variance (ANOVA) and compared by F-test using the STATISTICA 20 (Stat Soft, Inc., USA) software package. The final values have been presented as mean ± standard deviation. Differences were considered to be significant when $P<0.05$.

2.10. Circular dichroism (CD) spectra

The circular dichroism (CD) spectra were measured on Aviv circular dichroism spectrometer model 215 (Aviv Instruments, NJ, USA). The far-UV (260-200 nm) and near-UV (320-250 nm) CD spectra were measured in 50mM Tris-HCl (pH 8.0) containing 0.15 M NaCl and 10 mM $CaCl_2$ at 25 ^{o} C. The protein concentration and optical path length were 0.2 mg ml^{-1} and 0.1 cm for far-UV CD spectra, and 0.4mg ml^{-1} and

1 cm for near-UV CD spectra, respectively.The buffer 50mM Tris-HCl (pH 8.0) with 0.15 M NaCl and 10 mM $CaCl_2$ was used as blank and its spectrum was subtracted to CD spectra of proteins. The results are expressed as molar ellipcity $[\theta]_\lambda$(deg cm^2 $dmol^{-1}$) by using a mean amino acid residue weight (MWR) of 110. The CD units used are the mean residue ellipticity for CD spectra in the far-UV range and the molar ellipticity for CD spectra in the near-UV range. The ellipticities are calculated from the following relations, $[\theta]_\lambda$Far = (θm × 100 MWR)/(*c.l.n*) and $[\theta]_\lambda$Near = (θm × 100 MWR)/(*c.l*), where θm is the measured ellipticity in degrees wavelength λ, *c* is the protein concentration in mole per liter, *l* is the light path length of the cell in centimeter and *n* is the number of residues.

3. Results

3.1. Mutant enzyme design and construction

PCR mutagenesis was used to generate truncated lipase in order to investigate the importance of the eight GGXGXDXUX motifs in addition to an entire extreme C-terminal motif (49 residues), of SML by its expression in *E.coli* cell without Lip-system (LipBCD), as well as their role in the activity and stability of the enzyme. To achieve the above objectives, selection of amino acid residues for knockout mutagenesis was done based on a 3D model of SML. This model was generated using a previously reported structure of *S. marcescens*lipase (PDB code 2QUA) as a template, which showed 98% sequence similarity with our enzyme. In the resulting model, location of the two beta-roll domains was detected. The last 128 residues of SML, which form the second β-roll, were chosen for mutagenesis and the SML-Δ128 truncate was constructed. The C-terminal amino acid sequences of SML and its mutant protein constructed in this study are summarized in Fig. 1. Eight out from the thirteen the thirteen repetitive sequences and entire extreme end of the C-terminal motif is deleted. According to our model (as well as the used template crystal structure), these parts contribute to forming the parallel β-sheets. The performance of the mutation and the absence of PCR-generated random mutations were verified by DNA sequencing. The analysis of the sequences showed that mutation was successfully accomplished. Determination of the nucleotide sequence revealed that the SML is similar in size to PML and both are composed of 614 and 619 amino acid residues, respectively. The comparison of the amino acid sequence alignment of SML (accession no. AGT95802) with PML (accession no. BAA84997) in the C-terminal region, (Fig. 2), showed that the SML has similarity of 66.8 % to PML where a putative non-residue GGXGXDXUX sequences motif is repeated. These conserved sequences are partly degenerated, and further, the seventh nine-residue sequence motif has a different sequence in comparison to other sequences. The SML-Δ128 lacks the 21% amino acid sequences in comparison to SML (residues 487-614) and therefore contains five repetitive sequences (residues 374-418) like SML-WT in addition the 68-residue (residues 418-486).

3.2. Expression and purification of wild- type (full- length) and truncated forms of SML in *E. coli*

The proteins were overexpressed in *E. coli* BL21 cells transformed with PET-28a (+) by use of a T7 promoter-driven protein expression system in *E. coli* cells inducible with IPTG. The expression level of SML-Δ128 was comparable to that of the SML- WT as determined using SDS-PAGE (Fig. 3). The inclusion bodies, solubilized, and purified in the presence of 6M Gn-HCl or 8 M urea, and refolded in the presence of the Ca^{+2} ions. The two recombinant the wild- type and truncated SMLs, contained six histidine residues in N-terminal region of the protein, were then purified using metal-affinity chromatography on Ni-NTA resin column. Each of the both histidine-tagged lipases bound to Ni-NTA resin was eluted with imidazole. Remaining imidazole from the elution was removed by dialysis against 50mM Tris-HCl. After protein purification, the recombinant lipases band were for SML-WT and SML-Δ128 approximately 65 kDa and 52kDa on SDS-PAGE, respectively (Fig. 3).These purified proteins were used for further biochemical characterization.

3.3. Effect of pH and Temperature on lipase activity

The effects of pH and temperature on the SML-WT (wild-type) and the SML-Δ128 (truncated) were determined. Various buffers at different pH values were employed for evaluating the optimum pH of the full-length and truncated SML. The optimum pH values for enzymatic activity were determined between pH 7.0 and 9.0, with optimal pH 8.0, for SML- WT and between 8.0 and 9.0, with optimal pH 8.0 for SML-Δ128 (Fig. 4a). The optimum temperature for enzymatic activity of the wild-type and truncated SML was found at 42 and 37 ^{o} C, respectively (Fig. 4b) when they were assayed with 50 mM Tris-HCl buffer (pH 8.0) in the presence of 10 mM $CaCl_2$ and *p*-nitrophenyl laurate (C_{12}) as the substrate.

3.4. Substrate specificity

The substrate specificity of both enzymes, were analyzed towards several *p*-nitrophenyl esters of different chain lengths having alkyl chain lengths of C_2, C_4, C_{12}, and C_{16}, using spectrophotometrically under the assay conditions. As shown in Fig. 5 the highest hydrolytic activity was obtained with C_4 and C_{12} *p*-nitrophenyl esters, with a remarkable preference of toward *p*-nitrophenyl laurate (C_{12}) whereas with reduce was the activities toward C_2 and C_{16} esters.

3.5. Kinetic parameters

Kinetic parameters for both enzymes were obtained by measuring rates of hydrolysis of different concentrations of *p*NPC_{12} as described in Methods. The Kinetic data were plotted (Fig.6). The specific activity of the SML-WT and SML-Δ128 were 309 ± 41 and 1265 ± 119 U/mg, respectively (Table 1). Approximate 4-fold and 3.7-fold increases in specific activity and turnover rate (*k*cat), respectively, was obtained for the truncated enzyme compared to the value for the wild-type enzyme. The catalytic efficiency (*k*cat/*K*m) of SML-Δ128 was 2.4-fold higher than that of the wild-type enzyme (Table 1). These results indicated that the protein truncation for ~13 kDa at the C-terminal region of SML resulted in a substantial improvement.

3.6. CD spectra

To inspect whether the total structure of SML is changed by the mutation The CD spectra of the SML-WT and SML-Δ128 were measured in 50 mM Tris- HCI (pH 8.0) containing 10 mM $CaCl_2$, at 25°C as previously described. The far-UV CD spectrum reflects the secondary structure of the protein, whereas the near-UV CD spectrum reflects the local conformation around Tyr (Tyrosine) and Trp (Tryptophan) .The far-UV CD (Fig. 7A) and the near-UV CD (Fig. 7B) spectra of the SML-Δ128 were slightly different to those of SML- WT, suggesting that the protein secondary structures and the three-dimensional environments of the aromatic residues in the protein was slightly changed by the C-terminal truncation. The far-UV CD spectrum of the SML-Δ128 displayed a shift (θ) in the depth at 210-230 nm followed a shoulder at spanning 215-225 nm that it was a marked difference than the SML- WT, as well, the near-UV CD spectrum of the SML-Δ128 which exhibited different from that of the SML- WT, probably because the local conformation of the aromatic residues due to removal of 128 residues.

4. Disscution

As described above family I.3 lipases are clearly different from group I and II lipases in that they (i) do not contain a typical N-terminal signal sequence, and (ii) do not contain Cys-residues [27] . These lipases are represented by lipases from *S.marcescens* and *P.fluorescens* with an identity of about 65% over virtually the entire length of primary protein sequence that are secreted by a one step pathway using an ATP-binding cassette exporter (ABC) system, (or type I secretion system (T1SS) [8, 28]. Secretion by this system occurs in a single step, directly from the cytoplasm to the extracellular, by passing the periplasm. Furthermore family I.3 lipases have been shown to be secreted by heterologous T1SS, reconstituted in *E. coli* or in their original bacterial hosts [29]. Family I.3 lipases can be secreted by heterologous T1SS from *S. marcescens* lipase (LipBCD or Lip system) reconstituted in *E. coli* or in their original bacterial hosts when the genes encoding family I.3 lipases and the Lip system are co-expressed in *E. coli* [13, 27]. The *lipBCD* genes encoding the Lip system, which is composed of LipB (an ABC protein), LipC (a membrane fusion protein), and LipD (an outer membrane protein) promotes the secretion of portions [8, 29]. The SML coded by the *lipA* gene has no conventional N-terminal signal sequence commonly required for the secretion of lipase, , but it contained an extreme C-terminal motif consisting of a hydrophobic five-residue sequence that can be recognized by an ABC-transporter system (Lip system) [13]. Family I.3 lipases expressed in two ways (a) in original bacterial hosts and (b) co-expressed with Lip system reconstituted in *E. coli.* The role (s) of the repetitive sequences for both the mentioned routes may be different and remains to be elucidated. It has been proposed that the repetitive sequences in PML are important for the stability in *E. coli* with Lip-system because both the amount of the mutant protein secreted into the external medium and accumulated in the cells decreased , as the number of repetitive sequences in the mutant protein was reduced [21]. Various studies on the role of these repetitive sequences, when is used via the Lip-system, on different proteins have been shown that to be required for secretion [19] and or they were not required for secretion, but were needed for activity and stability [30]. It has been proposed that in this model (with Lip-system), mutant proteins lacks of the repetitive sequences may be highly unstable and rapidly degraded by cellular protease before they recognized by the ABC transporters for secreted into the external medium [21]. With this interpretation , present work is the first study on engineering and modeling the *lip*A gene of *Serratia marcescens* belonging to family I.3 lipase, hence a SML-WT derivative, SML-Δ128, was successfully constructed by deletion mutation of 128

residues at the C-terminal region. The genes encoding the SML-WT and the SML-Δ128 proteins without Lip-system (LipBCD), were overexpressed in *E.coli* with a high yield using the pET system as inclusion bodies, then solubilized in buffer containing 8 M urea, and refolded in the presence of Ca^{2+} ion. However, we found that the conformation and activity of the SML-Δ128, whenever was refolded in the absence of the Ca^{2+} ions were different than refolding in the presence of the Ca^{2+} ion (data not shown), suggesting that the presence and/or the absence of Ca^{2+} ions for enzyme activity the SML-Δ128 were different from those of the SML-WT. It seems that similar PML, the repetitive sequences play such a role in SML. As previously shown, PML exhibits Ca^{2+}-dependent activity and at least five from thirteen repeats are required for to be active (bind Ca^{2+} ions, and to fold into a putative β-roll motif) [16, 21]. These results suggest that folding of family I.3 lipases into their active conformation requires the presence of at least five functional repetitive sequences and the number of repetitive sequences can be reduced to five without affecting the enzymatic activity. Our results indicate that truncation of 128 amino acid residues at the C-terminal region of full-length lipA was not involved its enzyme activity and its folded structure was maintained for activity as the resultant SML-Δ128, contains five of the thirteen motifs. Kinetic analyses of the truncated SML in this study, namely, SML-Δ128, revealed that it possessed more enzymatic activity than the wild-type enzyme. The specific activity of of the full-length (SML-WT) and truncated (SML-Δ128) forms of SML were 309 ±41 and 1265 ±119 U/mg, respectively (Table 1). An approximately 4-fold and 3.7-fold increase in specific activity and turnover rate (*k*cat), respectively were obtained for the truncated enzyme, as compared to the value for the wild-type enzyme. This observation is similar to the results reported on different enzymes with the C-terminal truncated region [31-33], as the deletion at C-terminal region led to a significant increase in enzymatic activities. From obtained results of enzymatic activity analysis, we can suggest that the SML could tolerate extensive modification in the C-terminal region. This may be due to the following reasons: (i) there are no cysteine residues in the deduced amino acid sequence of SML. Cysteines are often participated in the formation of disulfide bonds in proteins, and proteins without cysteines are usually more flexible because of the lack of disulfide bonds [10, 34] therefore , it has been noted that SML protein have no cysteine , as well as lack of disulfide bonds thereby a possible to increase its enzymatic activity of SML-Δ128 is that it may more readily allow the conformational change that accompanies interfacial activation , (ii) SML has a large extension at the C-terminal part and has a higher molecular mass than lipases from families I.1 and I.2 , with about 614 amino acids and Mr of 65000. It may be suggested that due to the smaller size of SML-Δ128 compared with SML-WT, the contact surface for substrate and molecular interactions would more easily occur, due to increased flexibility , (iii) the active-site is located close to the N terminus of the protein , in addition the C-terminal 128 residues of the full-length lipA, forms an independent domain from the N-terminal domain (The second β-roll domain) therefore, SML-WT could tolerate extensive modification in the C-terminal extreme of the protein without seriously affecting on enzyme activity. The various proteins, such as metalloproteases from *S. marcescens*, [17, 35] *P. aeruginosa* , [36-38] lipase from *P. fluorescens* [39] and *S. marcescen*, [14] that been determined using crystal structures revealed that, these consist of an the N-terminal catalytic domain and the C-terminal β- sandwich domain. It have been proposed that the C-terminal domain consists a series of a glycine-rich sequence motif GGXG(N/D)DX(U)X (X represents any amino acid, and U represents any large hydrophobic residue such as Ile (I), Leu (L), Val (V), Phe (F) , Tyr (Y)), representing the main Ca^{+2} -binding sites of the protein at the C-terminal end of each protein, called a parallel β-helix or parallel β-roll where the first six residues of each nine-residue sequence motif form a turn which binds Ca^{+2} ions and the remaining three residues build a short β-strand. One turn of this helix consists of two consecutive nine-residue motifs and each Ca^{+2} ion is bound between a pair of these loops that usually too tightly bound [7, 17]. The repetitive sequences of and SML and PML are not consecutive but are interrupted by a large peptide insertion. The number of repeats varies between different proteins , [40, 41] for example, the C-terminal domain lipase from *S. marcescens* and *P. fluorescens* contain 12-14 repeats of nine-residue sequence motif that are laterally stacked together and 6–8 calcium ions bind [9, 14, 16, 18]. The absolute function of these tandem repeats remains somewhat vague, it have been proposed to be enhancers of protein secretion be secreted by an ABC-transporter system ,[42] and or acts as internal chaperones for protein folding [38]. It has been reported that in *S. marcescens* , a Ca^{2+} ion binds the lid helix, this Ca^{2+} ion is hexacoordinate with the

side chains of Gln120 , Asp153 (monodenate) and Asp 157 (bidentate), and main chain oxygen atoms of Thr118 and Ser 144 that in *S. marcescens* the Ca1 site, termed lid 1 , is formed. Besides the a Ca^{+2} ion as described, seven additional Ca^{+2} ion(s) at the Ca2 site were identified that six bound to the nine-residue sequence motifs and remaining one is coordinated by the side chains of Glu254 (monodentate), Asp276 (bidentate), and Asn285, main chain oxygen atom of Asn284, and two water molecules [7, 14, 29, 41]. The C-domain of SML, similar to that of PML, contains two the parallel β-roll domains consisting of the repetitive sequences, that together forming the so-called β-roll sandwich. The repetitive sequences of both SML and PML are not consecutive but are interrupted by a large peptide insertion, and further, two the parallel β-roll domains in SML and PML separated by a 72 and 74 residues spacer, respectively (Fig. 1 and Fig 2).The first β-roll domain of the SML and PML are located at spans residues 374–418 and 372–418, respectively, which containing five repetitive sequences G*X*G*X*D*X*U*X* motifs. These conserved sequences are somewhat degenerated. The first β-roll motifs SML and PML, similarly, the sixth aspartic acid residue (D) of nine-residue motif is mutated and is replaced by Asn (N) and hence, Ca^{2+}binding site is incomplete, consequently only binds three Ca^{2+} ions to them, also this part without the presence of water molecule i.e. has no coordination with water molecules. The second β- roll domain of the SML and PML are located ranging residues 491– 565 and 493-566, respectively, which contains eight, nine-residue motifs and binds five Ca^{2+} ions. Bottommost two Ca^{2+} ions which bind to this motif and have coordination with water molecules, while the remaining three do not, probably because which each Ca^{2+} ion binds between a pair of the loops formed by the nine-residue motifs. Unlike the first β-roll motifs, the second β-roll motifs the SML and PML are different, the sixth aspartic acid residue motif of the PML, which coordinates with the Ca^{2+} ion, is exactly conserved in all motifs. However, the space between the loops of the second and fourth consensus motifs is occupied by water molecule, lieu of Ca^{2+} ion while in the second β-roll motifs of the SML, the sixth aspartic acid residue motif is replaced by Ala (A), Asn (N), and Asn (N) in the second, fourth, and sixth motifs, respectively accordingly only three Ca^{2+} ions bind to the β-roll motif. Therefore, the first and second β-roll motifs contain at most three and six Ca^{2+}-binding sites, respectively. All probable Ca^{2+}-binding sites are surrounded by the Ca^{2+} ion in the first β-roll motifs of the SML and PML. In contrast, only three and five Ca^{2+}-binding sites are occupied by the Ca^{2+} ion in the second β-roll motifs of the SML and PML, respectively [23]. Similar studies have also been suggested that some family I.3 lipases contain only six repetitive sequence [43] furthermore, in PML five repetitive sequence are enough to maintain the functionality and structural integrity and neither C-terminal truncation nor single mutation five repetitive sequence at the C-terminal regions seriously affects the enzymatic activity of PML [21]. It has previously shown on PML, the parallel β-sheet of one side of the first β-roll motif is extended to form a long parallel β-sheet with three β-strands of the lipase domain, therefore the first β-roll motifs is probably needed to promote folding of the lipase however it contains only five repetitive sequence motifs [16]. Till now limited information is available regarding the effect of truncation C-terminal on the enzyme activity of family I.3 lipases. From the results of the lipase activity of rSML-WT and rSML-Δ128 proteins, shown that the truncation of the C-terminal region not affects on enzymatic activity of SML. In this truncation removes C-terminal half, including 8 from 13 repetitive sequences with 49 residues, therefore the mutation of either one by one of these repetitive sequences, the remaining five, in SML is recommended to illuminate their role (s) and the effects on enzyme activity. As a study for PML, by removing 7,8,11, and/or all the repetitive sequences has been reported that the number of repetitive sequences in PML can be reduced to five without seriously affecting the enzymatic activity, but removal of an additional single repetitive sequence, leads inactivates the protein [21] as well as, when 19 residues were deleted at an extreme the C-terminal region, (PML-Δ19), showed the enzymatic activity. It has also been reported for SML that a hydrophobic five-residue sequence motif (VTLIG), which is located upstream of an extreme C-terminal motif, is required for secretion of the protein by type 1 secretion system (T1SS) [43]. The comparison of the amino acid sequences this motif in both SML and PML shows that is similar, except the forth residue which are Ile_{599} (I) and Val_{602} (V) for SML and PML, respectively (Fig. 2). Therefore, in here this motif was deleted. It has previously shown that it is probably required to separate the C-terminal secretion signal from the passenger proteins and thereby facilitate identity of the signal by an ATP-binding cassette (ABC) transporter and furthermore, hydrophobic interactions may

be responsible for recognition of a secretion signal by Lip-system ,and finally are secreted into external medium [16, 23, 29]. The far- and near-UV CD spectra of the SML are slightly changed by the truncation of an extreme the C-terminal region. The far-UV CD spectra of SML-WT and SML-Δ128 when were compared, it was observed that a shift (θ) in the depth at 210-230 nm , a shoulder at spanning 215-225 nm , with decrease in the number of repetitive sequences in the protein (Fig.7A) Likewise, according to the same study [21] suggested that the last three residues of each repetitive sequence form a β-strand in a β-roll structure, hence thereby the decrease in the depth of the trough at 210-230 nm may be due to a lowerβ-roll content in the protein as well, at least partly account for this difference. Likewise, the near-UV CD spectrum of the SML-Δ128 was slightly different from those of the SML-WT at 260–320 nm. The same as previously suggested [18, 23] several aromatic residues, such as Trp506, Phe508, Phe526 , Phe544, Phe546 , Phe550 , Tyr556 , Phe558 , Phe566 Tyr579 , Phe591 and Phe606 that are located in the vicinity of an extreme the C-terminal region of the SML-WT, therefore the conformations of these residues by removal may be changed as shown in Fig. 7B.

5. Conclusion

In conclusion, an active lipase truncated microbial has been obtained in this study. As shown truncation of 128 C-terminal residues creates a mutant protein and when was overexpressed in *E.coli* strain, without using the Lip-system, significantly an increased enzymatic activity was observed. Purification and biochemical characterization of this protein indicate that this mutation does not seriously affect the structures and functions of SML. Thus, this work can provide a significant contribution to further research on SML to create a smaller enzyme potentially useful for industrial applications.

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Figure legends

Fig. 1 Schematic representation of the primary structures of SML. In N-terminal catalytic domain, the position of the conserved amino acid residues in GXSXG motif, in which the active-site is located in Ser_{207}, Asp_{256} and His_{313} residues that form a catalytic triad, are shown. In C-terminal domain, hydrophobic the nine-residue GGxGxDxux sequence motif that repeated five times at 374–418 and eight times at 487-565 is indicated. A putative secretion signal, hydrophobic five-residue sequence motif (VTLIG), which is located at upstream an extreme C-terminal region is also shown. The range of the amino acid sequence (Ala_{487}-Ala_{614}), which is deleted in the SML in this study, are denoted.

Fig. 2 The comparison of alignment of amino acid sequences in the C-terminal region of lipases from *S. marcescens* (SML) and *Pseudomonas sp.* MIS38 (PML) has been performed using the program CLUSTALW2. For the SML and PML sequences, the identical residues are shown by lines. Gaps are shown by dashes. The nine-residue GGxGxDxux sequences motifs are represented by solid boxes. A hydrophobic five-residue sequence motif, which has been proposed to be a secretion signal , is also shown as a box in an extreme C-terminal region that in both SML and PML is similar, except the forth residue which are Ile_{599} (I) and Val_{602} (V) for SML and PML, respectively. The accession numbers of proteins for the SML and PML sequences are in GenBank AGT95802 and BAA84997, respectively.

Fig. 3 SDS-PAGE (12% gel) analysis of the full-length (SML-WT) and truncated (SML-Δ128) forms of SML that were overexpressed in the *E. coli* BL21(DE3) cells transformed with plasmid PET-28a(+) as described in Materials and Methods. The gel was stained by Coomassie brilliant blue R-250. Lane M, molecular weight marker (kilo Daltons, kDa) ; lane 1, purified SML-WT ; lane 2, precipitate of cell lysate (Inclusion Body) SML-WT induced by 1mM IPTG; lane 3, cell lysates without induction (control); lane 4 , purified SML-Δ128; lane 5, precipitate of cell lysate (Inclusion Body) SML-Δ128. Recombinant proteins are indicated by arrows which are approximately 65kD and 52kD for SML-WT and SML-Δ128, respectively

Fig. 4 Effects of pH and temperature on enzyme activity of the full-length (SML-WT) and truncated (SML-Δ128) forms of SML. (a) Effect of pH on lipase activity was determined at 37 and 42 °C for SML-Δ128 and SML-WT, respectively over a pH range from 4.0-12.0 in the presence of 10 mM $CaCl_2$ by using *p*NPC_{12} as the substrate. b) Effect of temperature on lipase activity was measured at pH 8.0 by using *p*NPC_{12} as the substrate in temperature range of 30–50 ^{o} C under standard conditions. Relative activity was calculated by assuming percentage of maximal activity, defined as 100%, at optimum pH and temperature for both enzymes. The SML-WT (Circle filled) and the SML-Δ128 (Triangle filled) are shown. Date presented were the average values of three independent experiments and the error bars indicated standard deviations.

Fig. 5 Substrate specificity of the purified the SML-WT (wild-type) and the SML-Δ128 (truncated), towards several *p*-nitrophenyl esters of different chain lengths are shown. Relative activities are expressed as the percentage of that of *p*-nitrophenyl laurate (C_{12}), which was taken as 100%. Date presented were average values of three independent experiments and the error bars indicated standard deviations.

Fig. 6 The Michaelis-Menten and Lineweaver-Burk plots (a) the SML-WT (wild-type) and (b) the SML-Δ128 (truncated), were measured using *p*-nitrophenyl laurate (C_{12}) as substrates at a series of concentrations. Kinetic analyses were determined in 50 mM Tris HCl buffer (pH 8.0) in the presence of 10 mM $CaCl_2$ at 42 ^{o}C and 37 ^{o}C for the SML-WT and the SML-Δ128, respectively. Date

presented were the average values of three independent experiments and the error bars indicated standard deviations.

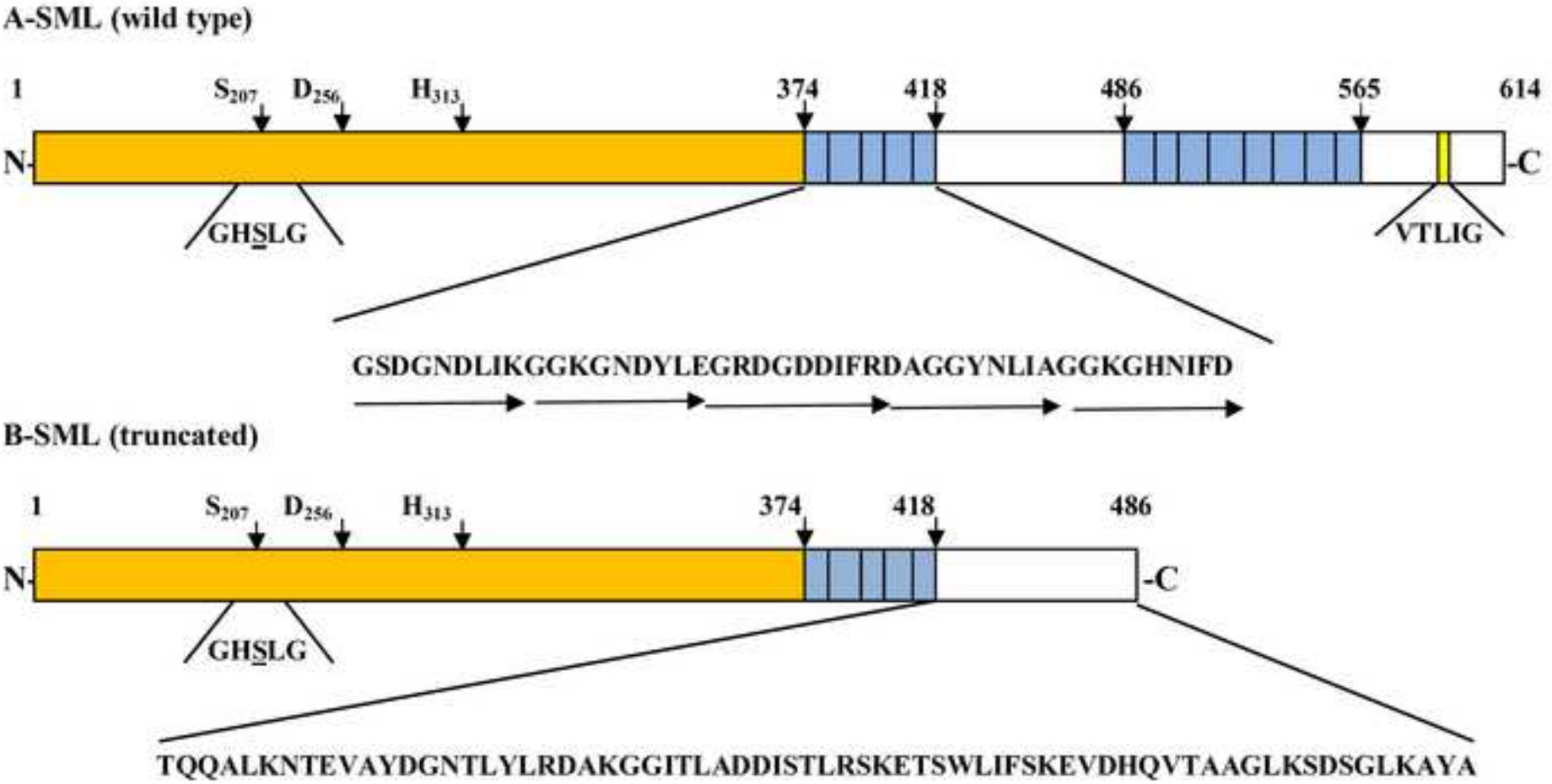
Fig. 7 The CD spectra of SML-WT (wild-type) and SML-Δ128 (truncated). The spectra of the SML-WT and SML-Δ128 proteins were measured in 50 mM Tris- HCI (pH 8.0) containing 10 mM $CaCl_2$, at 25°C as described in Materials and Methods Section. (A) The far-UV CD spectra of SML-WT (Circle filled) and SML-Δ128 (Square filled) are shown. (B) The near-UV CD spectrum of SML-WT, (thick line), and SML-Δ128, (thin line), which displayed difference from each other, are shown.

Table1. The kinetic parameters of the SML-WT and the SML-Δ128

Lipase	Specific Activity (Umg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \times mM^{-1}$)
Wild type (SML)	309.6 ±41.7	0.65	1.66
Truncated (SML-Δ128)	1265.7±119.1	2.43	3.97

Enzyme assays were performed in 10 mM $CaCl_2$ with 50 mM Tris HCl buffer (pH 8.0) by using pNPL(C_{12}) as the substrate. Data were mean ± SE from three independent experiments. The significance of differences was determined by one-way Anova followed by Tukey's test.

Figure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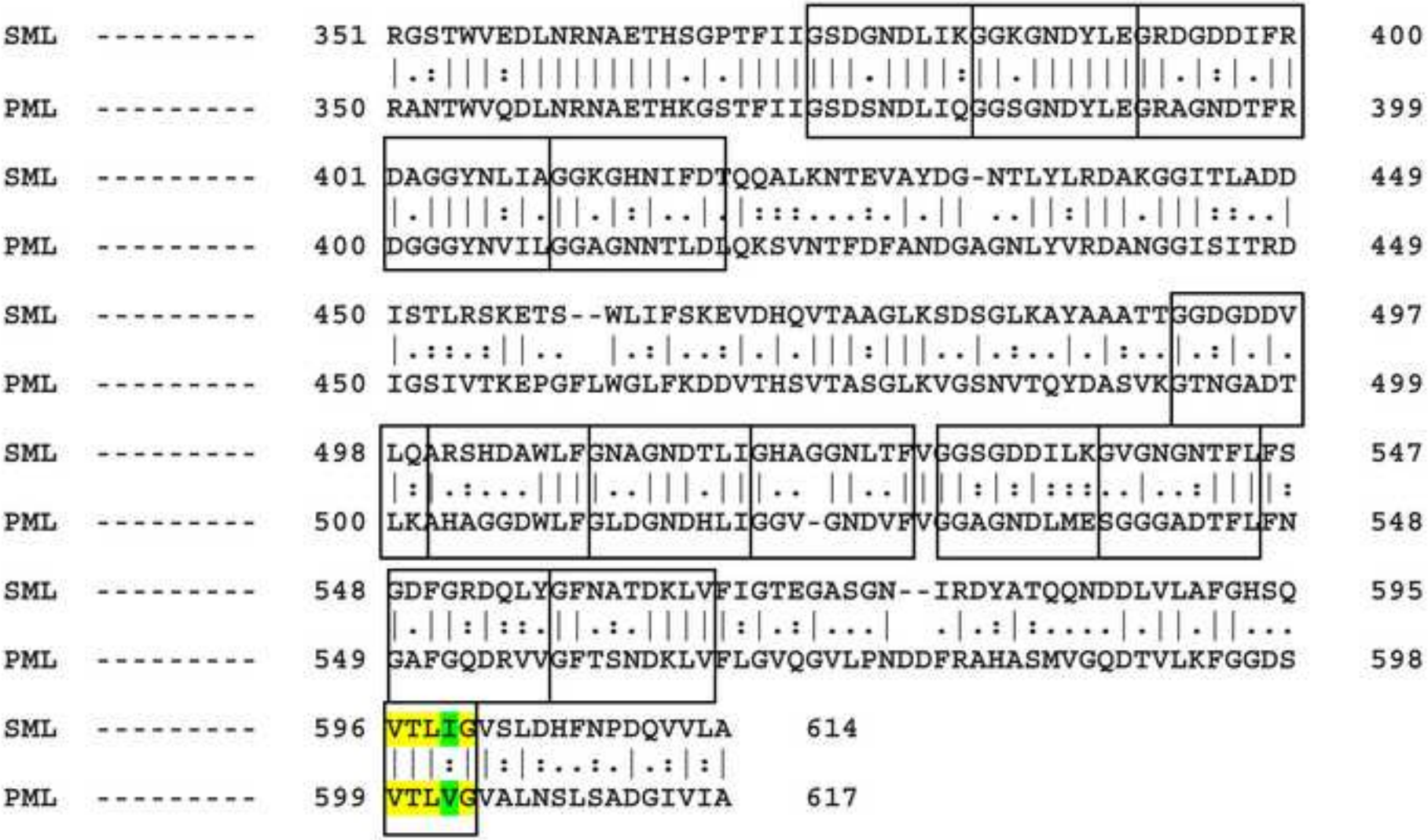
D256
H313
374
418
486
565
614
N-
-C
GHSLG
VTLIG
GSDGNDLIKGGKGNDYLEGRDGDDIFRDAGGYNLIAGGKGHNIFD
B-SML (truncated)
1
S207
D256
H313
374
418
486
N-
-C
GHSLG
TQQALKNTEVAYDGNTLYLRDAKGGITLADDISTLRSKETSWLIFSKEVDHQVTAAGLKSDSGLKAYA

Figure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PML --------- 350 RANTWVQDLNRNAETHKGSTFIIGSDSNDLIQGGSGNDYLEGRAGNDTFR 399
SML --------- 401 DAGGYNLIAGGKGHNIFDTQQALKNTEVAYDG-NTLYLRDAKGGITLADD 449
PML --------- 400 DGGGYNVILGGAGNNTLDLQKSVNTFDFANDGAGNLYVRDANGGISITRD 449
SML --------- 450 ISTLRSKETS--WLIFSKEVDHQVTAAGLKSDSGLKAYAAATTGGDGDDV 497
PML --------- 450 IGSIVTKEPGFLWGLFKDDVTHSVTASGLKVGSNVTQYDASVKGTNGADT 499
SML --------- 498 LQARSHDAWLFGNAGNDTLIGHAGGNLTFVGGSGDDILKGVGNGNTFLFS 547
PML --------- 500 LKAHAGGDWLFGLDGNDHLIGGV-GNDVFVGGAGNDLMESGGGADTFLFN 548
SML --------- 548 GDFGRDQLYGFNATDKLVFIGTEGASGN--IRDYATQQNDDLVLAFGHSQ 595
PML --------- 549 GAFGQDRVVGFTSNDKLVFLGVQGVLPNDDFRAHASMVGQDTVLKFGGDS 598
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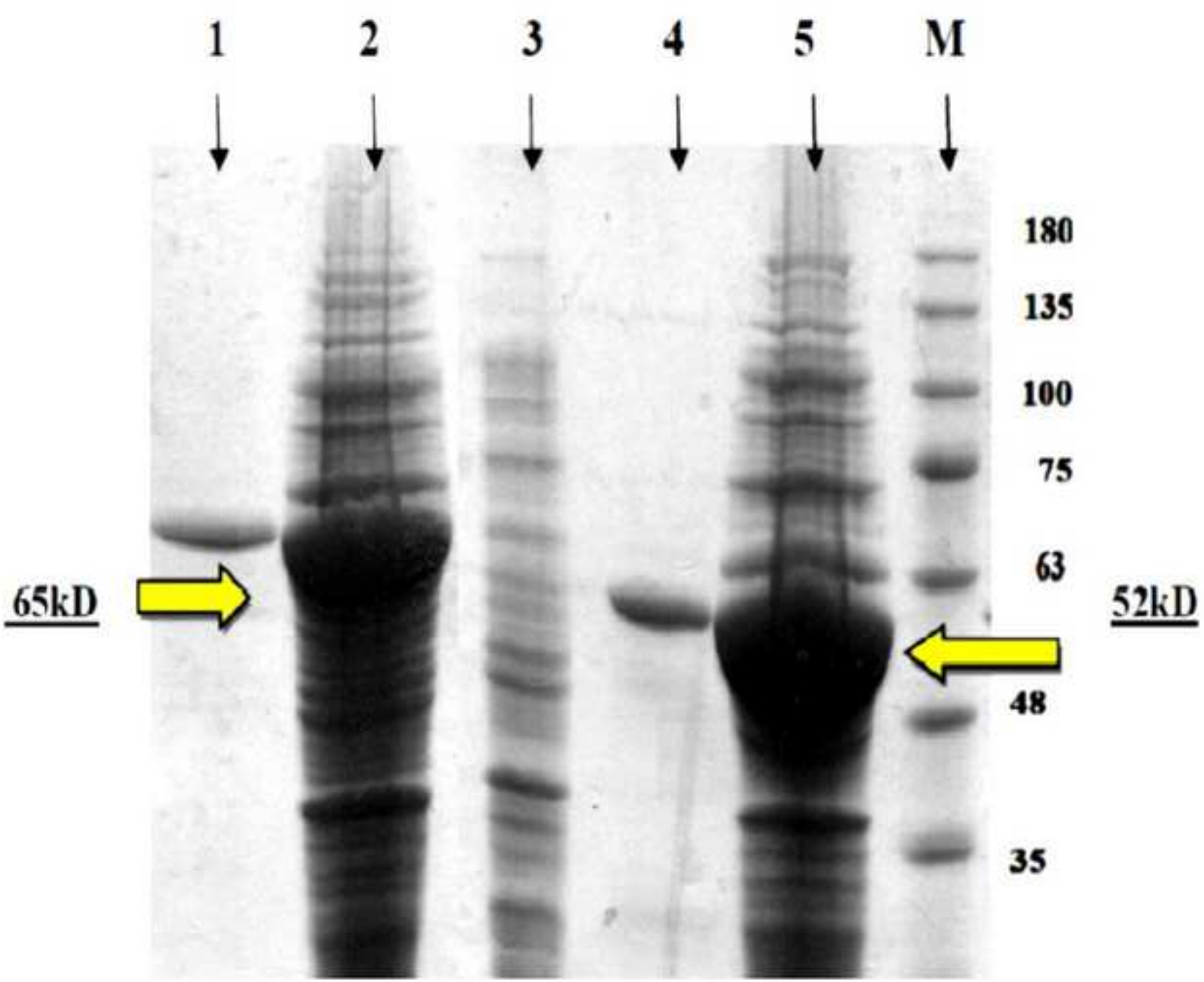
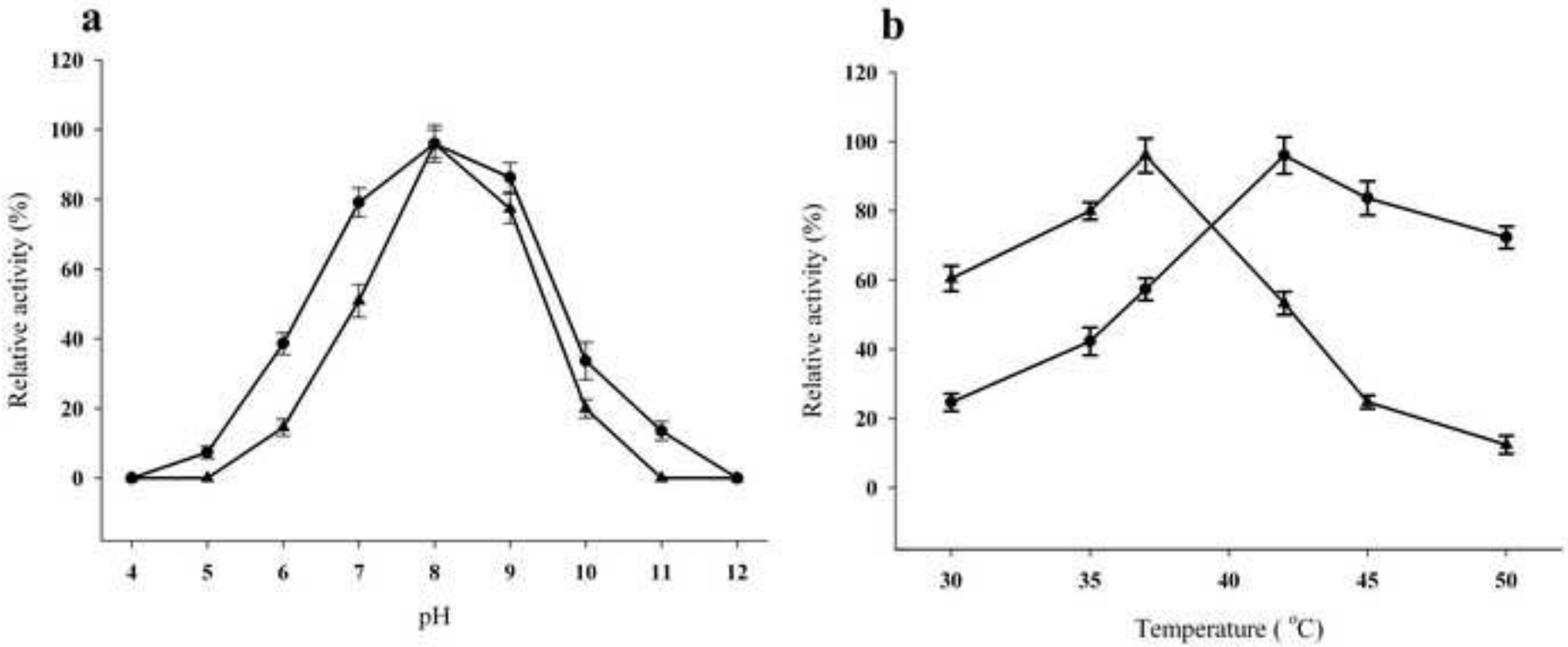
PML --------- 599 VTLVGVALNSLSADGIVIA 617

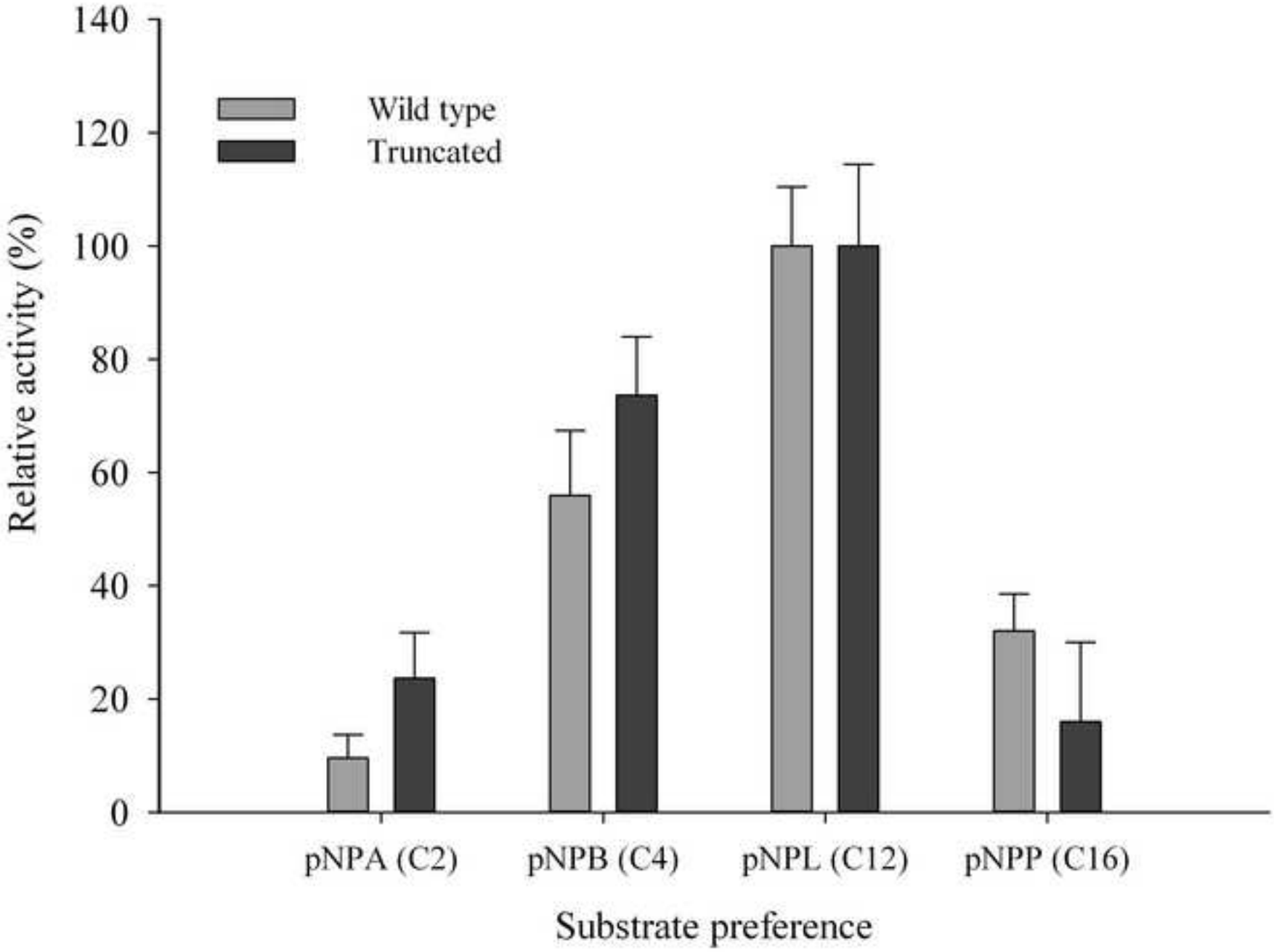
Figure

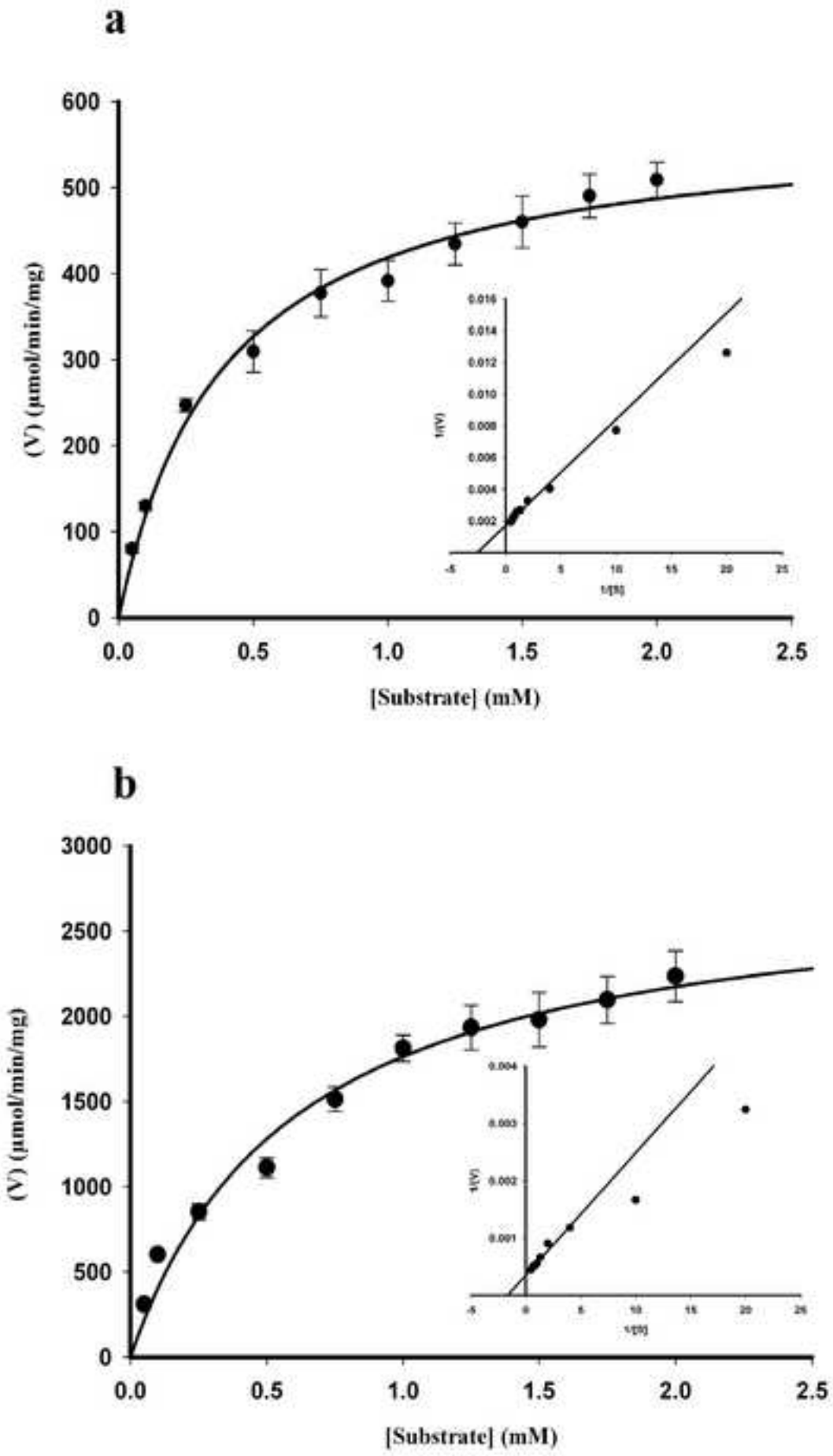


Figure

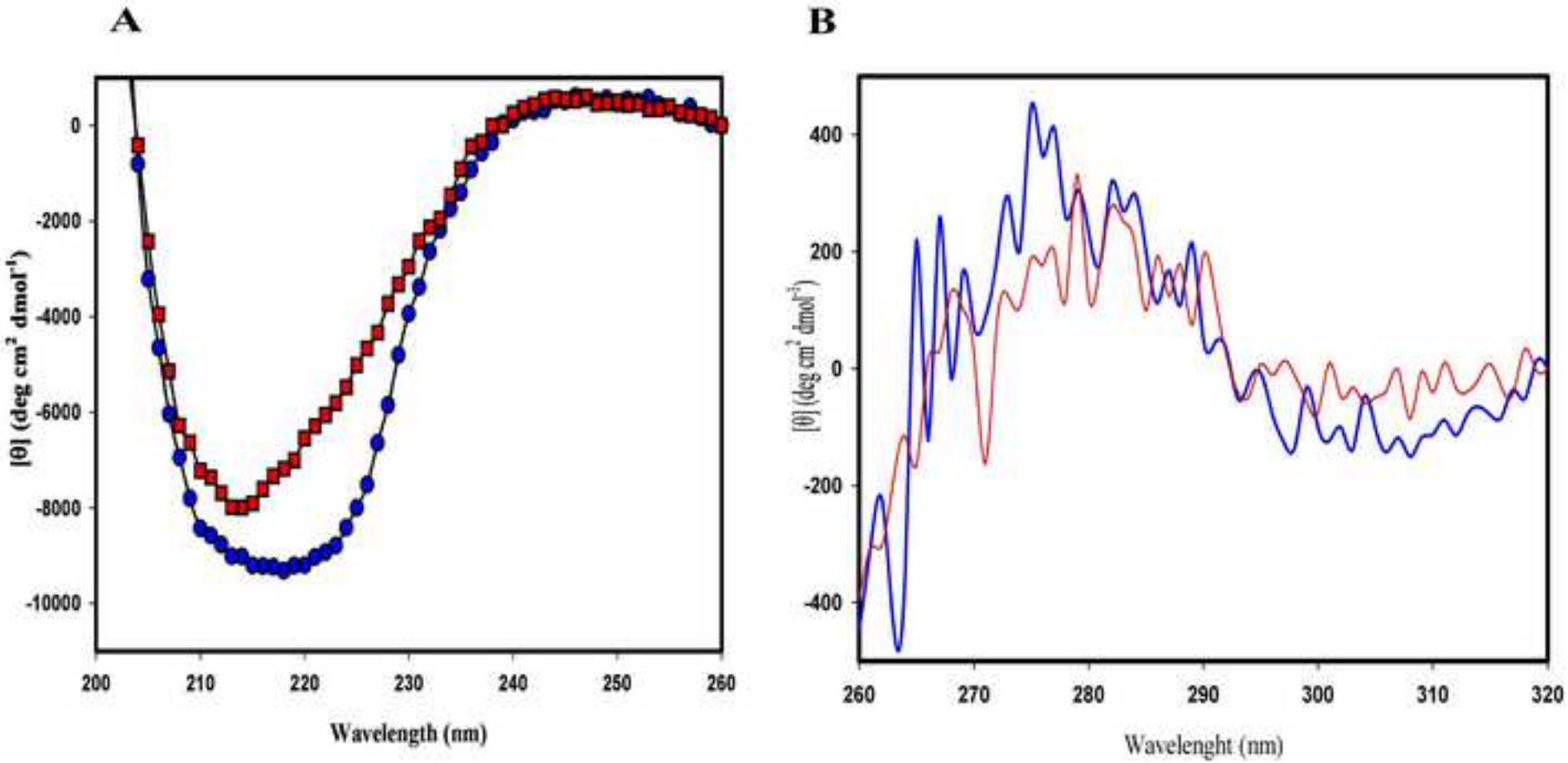

a
Relative activity (%)
120
100
80
60
40
20
0
4
5
6
7
8
9
10
11
12
pH
b
Relative activity (%)
120
100
80
60
40
20
0
30
35
40
45
50
Temperature (°C)

Figure


140
120
100
80
60
40
20
0
Relative activity (%)
Wild type
Truncated
pNPA (C2)
pNPB (C4)
pNPL (C12)
pNPP (C16)
Substrate preference



Figure


A
0
-2000
-4000
-6000
-8000
-10000
[θ] (deg cm² dmol⁻¹)
200
210
220
230
240
250
260
Wavelength (nm)
B
400
200
0
-200
-400
[θ] (deg cm² dmol⁻¹)
260
270
280
290
300
310
320
Wavelenght (nm)