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Biochemical and molecular characterisation of a thermoactive, alkaline and detergent-stable lipase from a newly isolated *Staphylococcus aureus* strain

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ABSTRACT

A newly soil-isolated *Staphylococcus aureus* strain secretes a non-induced lipase in the culture medium. The extracellular lipase from *S. aureus* (SAL3) is purified to homogeneity. The purified enzyme is a tetrameric protein (180 kDa) corresponding to the association of four lipase molecules. The 15 N-terminal amino acid residues showed a high degree of homology with other staphylococcal lipase sequences. The part of the gene encoding the mature SAL3 is cloned and sequenced. The deduced polypeptide sequence, corresponding to the mature SAL3, was very similar to the mature *Staphylococcus simulans* lipase sequence with two additional amino acid residues (LK) at the N-terminus of SAL3. The lipase activity is maximal at pH 9.5 and 55 °C. The specific activity of about 4200 U/mg or 3500 U/mg was measured using tributyrin or olive oil emulsion as substrate, respectively, at pH 9.5 and 55 °C.

In contrast to other staphylococcal lipases previously characterised, SAL3 is found to be stable between pH 5 and 12 after 24 h incubation. The enzyme retained 50% of its activity after 60 min incubation at 60 °C. This novel lipase is able to hydrolyse its substrate in presence of various oxidizing agents as well as some surfactants and some commercial detergents, then SAL3 can be considered as a good candidate for industrial and biotechnological applications.

1. Introduction

Lipases (EC 3.1.1.3) represent an important group of biotechnologically valuable enzymes [1]. They are widely distributed in nature. Although lipases have been found in many species of animals, plants, bacteria, yeast, and fungi, the enzymes from microorganisms are the most interesting because of their potential applications in various industries such as food, dairy, pharmaceutical, detergents, textile, biodiesel, and cosmetic industries and in synthesis of fine chemicals, agrochemicals, and new polymeric materials [2,3]. Bacterial lipases received much attention for their substrate specificity and their ability to function in extreme environments. The high-level production of microbial lipases requires not only the efficient overexpression of the corresponding genes but also a detailed understanding of the molecular mechanisms governing their folding and secretion [3,4].

Some of the industrially important chemicals manufactured from fats and oils by chemical processes could be produced by lipases under mild conditions with greater rapidity and better specificity [5,6]. The stereo-, regio- and enantiospecific behaviours of these enzymes have caused tremendous interest among scientists and industrialists [7].

In addition to their natural function of hydrolysing carboxylic ester bonds, lipases can catalyse esterification, interesterification, and transesterification in non-aqueous media.

Lipases are also important drug targets or marker enzymes in the medical sector. They can be used as diagnostic tools and their presence or increasing levels can indicate some kinds of infections or diseases [8].

Many staphylococci produce lipases which are released into the culture medium. Staphylococcal lipases were purified and biochemically characterised. They have the tendency to form aggregates [9–11] and they have a wide substrate specificity [12]. The activity of some enzymes appeared to be stimulated by calcium ions and inhibited in the presence of chelators such as EDTA or EGTA [13–16]. Some studies, based on sequence comparison or direct mutagenesis, have attempted to explain the biochemical dif-

Abbreviations: SAL3, *Staphylococcus aureus* lipase; SSL, *Staphylococcus simulans* lipase; SXL, *Staphylococcus xylosus* lipase; SAL NCTC8530, *Staphylococcus aureus* NCTC8530 lipase; SEL, *Staphylococcus epidermidis* lipase; SHL, *Staphylococcus hyicus* lipase; BSA, bovine serum albumin; CMC, critical micellar concentration; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid; HPLC, high performance liquid chromatography; NaDC, sodium deoxycholate; NaTDC, sodium taurodeoxycholate; PC, phosphatidylcholine; PCR, polymerase chain reaction; SDS/PAGE, sodium dodecyl sulphate/polyacrylamide gel electrophoresis; TG, triacylglycerol; TC_3, tripropionin; TC_4, tributyrin.

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ferences between staphylococcal lipases. The *Staphylococcus hyicus* lipase (SHL) is the first lipase family I.6 member of which the 3D-structure has been, recently, elucidated in an open conformation at 2.86 Å resolution [17]. This crystal structure explained the unusual phospholipase activity of SHL and its broad substrate specificity when compared to other staphylococcal lipases [17].

Reports of thermostable lipases from *Staphylococcus* sp. and active in alkaline conditions are not previously described. Also, practical applications of staphylococcal enzymes may be limited due to relatively lower stabilities and catalytic activities under conditions that characterise industrial processes: high temperatures, extremes of pH values or non-aqueous solvents. In the past years, intense efforts have been focused on the engineering of enzymes with altered properties or better performance for practical applications. Therefore, screening of new microorganisms with lipolytic activities could facilate the discovery of novel lipases. In this paper we report for the first time the production, the purification and the characterisation of a thermoactive, alkaline and detergent-stable lipase (SAL3) from a newly isolated *Staphylococcus aureus* strain. The N-terminal sequence of the SAL3 was determined and compared to the known staphylococcal lipases. The part of the gene encoding the mature lipase was cloned and sequenced.

2. Experimental

2.1. Chemicals

Tributyrin (99%, puriss) and benzamidine were from Fluka (Buchs, Switzerland); tripropionin (99%, GC) was from Jansen (Pantin, France); phosphatidylcholine, sodium deoxycholic acid (NaDC), sodium taurodeoxycholic acid (NaTDC), Tween 80, yeast extract and ethylene diamine tetraacetic acid (EDTA) were from Sigma Chemical (St. Louis, USA); all other detergents used (Ariel, Axion and Omino Bianco) were purchased locally; gum Arabic was from Mayaud Baker LTD (Dagenham, United Kingdom); acrylamide and electrophoresis grade were from BDH (Poole, United Kingdom); marker proteins and supports of chromatography used for SAL3 purification: Sephacryl S-200 and mono *S*-Sepharose gels were from Pharmacia (Uppsala, Sweden); PVDF membrane was purchased from Applied Biosystems (Roissy, France); trans-blot cell apparatus was from Bio-Rad (Paris, France); casein peptone was from Merck (Darmstadt, Germany); pH-stat was from Metrohm (Switzerland); oligonucleotides were synthesised by GENOME Express (Grenoble, France).

2.2. Bacterial strains, plasmids and media

S. aureus was grown in 17 g/l pancreatic digest of casein, 3 g/l tryptone (Difco), 2.5 g/l glucose, 5 g/l NaCl and 2.5 g/l K_2HPO_4, pH 7.

E. coli strain DH5α was used as a cloning host. *E. coli* strains were grown in Luria-Bertani medium, supplemented with 100 μg/ml ampicillin whenever plasmid maintenance was required. The plasmid pCR® 4Blunt-TOPO® (invitrogen corporation) was used as cloning vector for the gene part encoding the mature lipase.

2.3. Screening of lipolytic microorganisms

Initial screening of lipolytic microorganisms from various Tunisian biotopes was carried out using a plate assay in a medium containing triacylglycerol and the fluorescent dye Rhodamine B [18]. The solid medium contains 1‰ olive oil, 1% nutrient broth, 1% NaCl, 1.5 g agar and 1‰ Rhodamine B. The culture plates were incubated at 37 °C, and colonies giving orange fluorescence halos around them, upon UV irradiation, were regarded as putative lipase producers [18].

After extensive screening of lipase producers, only one bacterial colony, isolated from the soil of an olive oil extraction industry, continued to give a positive signal when commercial detergent (1%) was added to the solid medium described above. The identification of this strain has been kindly determined by Dr. Hafedh Dhouib (Centre de biotechnologie de Sfax, Tunisia). The biochemical properties and the morphological aspect of this microorganism showed 99.7% identity to *S. aureus*.

2.4. Culture conditions

S. aureus was incubated overnight at 37 °C and 200 rpm in 1-l shaking flasks with 100 ml of medium A containing (l^{-1} distilled water): 5 g peptic digest of meat, 2 g yeast extract, 1 g beef extract powder, 5 g NaCl, pH 7. Overnight *S. aureus* cultures used as inocula were cultivated in 1-l shaking flasks with 100 ml of the medium B which contained (l^{-1} distilled water): 17 g pancreatic digest of casein, 3 g tryptone, 2.5 g glucose, 5 g NaCl, 2.5 g K_2HPO_4, pH 7. The culture was incubated aerobically during 36 h on a rotary shaker set at 200 rpm and at a temperature of 37 °C. Growth was followed by measuring the cultures OD at 600 nm.

2.5. Lipase activity determination

The lipase activity was measured titrimetrically at pH 9.5 and 55 °C with a pH-stat under standard conditions using tributyrin (0.25 ml) in 30 ml of 2.5 mM Tris–HCl pH 9.5, 2 mM $CaCl_2$, 1 mM NaDC or olive oil emulsion (10 ml in 20 ml of 9‰ NaCl pH 9.5, 2 mM $CaCl_2$, 2 mM NaDC) [19] as substrate. Lipase activity was also measured at pH 7 and 37 °C using TC_3 as substrate (0.25 ml TC_3) in 30 ml of 2.5 mM phosphate buffer pH 7, 2 mM $CaCl_2$. The olive oil emulsion was obtained by mixing (3 × 30 s in a Waring blender) 10 ml of olive oil in 90 ml of 10% GA. The catalytic constant (k_{cat}) values were calculated from the maximal velocities (V_{max}) values recorded at pH 9.5 and 55 °C using a pure tributyrin (TC_4), trioctanoin (TC_8) or triolein (TC_{18}) in presence of an excess of substrate concentration, using mass of 45 kDa for SAL3. The apparent Michaelis constant ($K_{M\,app.}$) values were determined graphically from double reciprocal Lineweaver-Burk plots of the kinetic data. Some lipase assays were performed in the presence of surface-active agents. When measuring SAL3 activity in the absence of $CaCl_2$, EDTA or EGTA was added to the lipolytic system.

Lipolytic activity was expressed as units. One unit corresponds to 1 μmol of fatty acid released per minute.

2.6. Phospholipase activity determination

The phospholipase activity was checked titrimetrically at pH 9.5 and 55 °C with a pH-stat using egg PC (0.2 g) mixed in 30 ml of 150 mM NaCl, 7 mM $CaCl_2$ and 3 mM NaTDC.

2.7. Determination of protein concentration

Protein concentration was determined as described by Bradford [20] using BSA as standard.

2.8. Procedure of SAL3 purification

500 ml of culture medium, obtained after 30 h of cultivation, were centrifuged for 15 min at 8500 rpm to remove the cells. The supernatant containing extracellular lipase was used as the crude enzyme preparation.

- Ammonium sulphate precipitation: The cell-free culture supernatant was precipitated using solid ammonium sulphate to 65% saturation. The pellet obtained after centrifugation (30 min at 8500 rpm) was dissolved in 10 ml of buffer A (20 mM sodium acetate pH 5.4, 20 mM NaCl, and 2 mM benzamidine). Insoluble material was removed by centrifugation at 13,000 rpm during 5 min.
- Heat treatment: The supernatant obtained (10 ml) was incubated at 55 °C during 3 min. Insoluble material was removed by centrifugation at 13,000 rpm during 5 min.
- Filtration on Sephadex G-100: The enzyme solution (10 ml) was applied to a Sephadex G-100 column (3 cm × 100 cm) previously equilibrated in buffer A at a rate of 30 ml/h. The fractions containing the lipase activity (eluted at a void volume) were pooled.
- Cation exchange chromatography: Active fractions eluted from Sephadex G-100 column were poured into a mono *S*-Sepharose cation exchanger equilibrated in buffer A. The column (2 cm × 30 cm) was rinsed with 400 ml of the same buffer. No lipase activity was detected in the washing flow. Adsorbed material was eluted with a linear NaCl gradient (300 ml of 20–500 mM in buffer A) at a rate of 45 ml/h. SAL3 activity was eluted between 170 and 220 mM NaCl.
- The fractions containing the lipase activity were pooled, concentrated and loaded on a column (1.6 cm × 110 cm) of gel filtration Sephacryl S-200 equilibrated in buffer A. Elution was performed at a rate of 20 ml/h.
- Active fractions eluted from Sephacryl S-200 were pooled, lyophilised and resuspended in 100 mM phosphate buffer, 150 mM NaCl, pH7. To determine the molecular mass of the native enzyme, 200 μg of the pure enzyme were poured into a gel filtration HPLC column Protein Bio. Sil SEC. 125 (300 mm × 7.8 mm) equilibrated in the same buffer. Elution was performed at 0.5 ml/min.

2.9. Analytical methods

Analytical polyacrylamide gel electrophoresis of proteins in the presence of sodium dodecyl sulphate (SDS-PAGE) was performed following the method of Laemmli [21]. Samples for sequencing are electroblotted according to Bergman and Jörnvall [22]. Protein transfer was performed during 1 h at 1 mA/cm^2 at room temperature.

2.10. Amino acid sequencing

The N-terminal sequence was kindly determined by Pr Hafedh MEJDOUB with automated Edaman's degradation, using an Applied Biosystems Protein Sequencer Procise 492 CLC [23].

2.11. DNA preparation and transformation procedure

Staphylococcal DNA was prepared as described previously [24]. Cells were lysed by the addition of lysostaphin (Sigma) at a concentration of 4.5 U/ml. *E. coli* supercoiled plasmid DNA was prepared by the modified alkaline lysis method [25]. *E. coli* was transformed using the $CaCl_2$ method. Enzymes for molecular cloning were obtained from Boehringer (Mannheim), BRL and Pharmacia LKB. Assay conditions were in agreement with suppliers recommendations.

2.12. Cloning of the mature lipase gene region

The gene part encoding the mature lipase was amplified by PCR from genomic DNA of *S. aureus* with primers, 5′-GATCGAATTCATATGTTAAAAGCGAATCAAGTACAACCACT-3′, and 5′-GATCGAATTCGGATCCTTAACTTGCTTTCAATTGTGTT-3′. The primers were predicted from the N-terminal sequence of the purified SAL3 and the complete genome of *S. aureus* Subsp. aureus MSSA475 (Accession number NC002953), respectively. SAL3 corresponds to the protein whose accession number is YP-042422.1 (Gene ID 2862193) described as lipase precursor. PCR was performed in a 0.2-ml Eppendorf tubes with a techne programmable oribloc PHC-1. The 50 μl PCR mixture contained 50 pmol of both primers, 20 pmol of each deoxynucleotide triphosphate, approximately 1 μmol genomic DNA as template, polymerisation buffer, and 5 U Taq polymerase (Amersham Pharmacia biotech). The thermal profile involved 35 cycles of denaturating at 94 °C for 45 s, primer annealing at 55 °C for 1 min, and extension at 72 °C for 2 min (10 min in the last cycle).

The PCR product (1.2 kb) was isolated and ligated into the *Eco*RI-linearised and dephosphorylated pCR® 4Blunt-TOPO® vector, using the pCR® 4Blunt-TOPO® blunt, according to the manufacturer's protocol (invitrogen corporation). Protoplasts of *E. coli* DH5α were transformed with the ligation mixture. The resulting recombinant plasmid was named pSAL3. The presence of the appropriate insert was determined by PCR and by digestion analysis. DNA products were analysed on a standard 1% agarose gel containing ethidium bromide. DNA sequences were determined using the dideoxynucleotide chain termination method according to a cycle sequencing protocol using thermosequenase (Amersham Pharmacia biotech).

The sequencing reactions were analysed with the DNA sequencer (Genom express, Grenoble, France). The sequencing was performed three times, using the recombinant vector (pSAL3) from three clones as template with T7 promoter primer and the U-19 mer primer (Amersham Pharmacia Biotech).

2.13. Nucleotide sequence access number

The nucleotide sequence determined in this study has been deposited in the GenBank database under access number EU035270.

2.14. Kinetic study

Lipase activities were measured as a function of various substrate (TC_4, TC_8 or TC_{18}) concentrations (0–40 mM). The Michaelis–Menten constant ($K_{M\,app.}$) and the maximum velocity (V_{max}) for the reaction with TC_4, TC_8 or TC_{18} as substrate were calculated by Lineweaver-Burk plot.

2.15. Effect of pH and temperature on SAL3 activity and stability

- SAL3 activity was tested in various buffers at different pH (5–10.5) at 55 °C. The pH stability of the lipase was determined by incubating the enzyme at different pH (3–12) for 24 h at room temperature. The residual activity was determined, after centrifugation, under standard assay method.
- The optimum temperature for the SAL3 activity was determined by carrying out the enzyme assay at different temperatures (25–60 °C) at pH 9.5. The effect of temperature on lipase stability was determined by incubating the enzyme solution at different temperatures (30–60 °C) for 60 min. The residual activity was determined, after centrifugation, under standard assay method.

2.16. Effect of surfactants, commercial detergents and oxidizing agents on SAL3 stability

The SAL3 sample (120 U from the ammonium sulphate precipitation fraction) was incubated in presence of 20 mM final concentration of Triton X-100, tween-80, sodium cholate or sodium

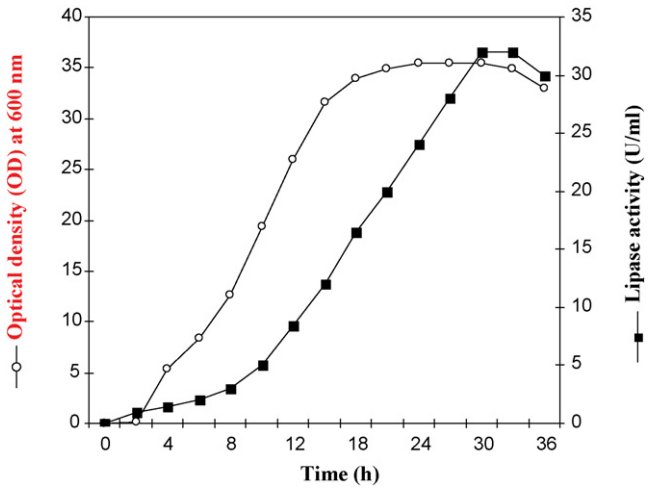

Fig. 1. The time courses cell growth of *S. aureus*, and lipase production. The culture was carried out at 37 °C in shaking at 200 rpm in presence or in absence of triacylglycerols or esters. Cell growth was monitored by measuring the absorbance at 600 nm.

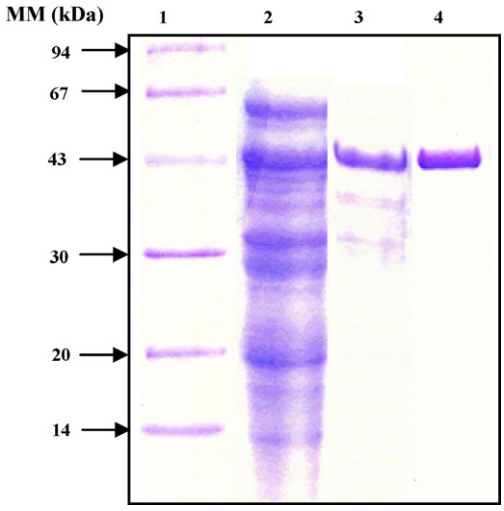

Fig. 2. Gel SDS/PAGE (12%). Lane 1, molecular mass markers; lane 2, profile of the SAL3 (20 μg) obtained after Sephadex G-100 chromatography; lane 3, profile of SAL3 (15 μg) obtained after Mono-S chromatography; lane 4, 10 μg of purified SAL3 obtained after Sephacryl S-200 chromatography.

taurocholate for 1 h at 40 °C. The effect on SAL3 stability of commercial detergents (1%, w/v for 1 h at 40 °C) or oxidizing agents (10%, v/v for 1 h at 40 °C) was also checked. Residual SAL3 activity was measured at pH 9.5 and 55 °C. To allow further comparison, the effect of surfactants, commercial detergents and oxidizing agents on other staphylococcal lipases (SSL or SXL) stability was also studied under the same experimental conditions. Residual SSL or SXL activity was measured as described previously [26,27].

3. Results and discussion

3.1. Production of lipase

The medium B (100 ml) was incubated with different amounts of inoculum from the overnight *S. aureus* culture. The maximum lipase production (30 U/ml of culture medium) was obtained after 30-h incubation, with an initial absorbance (OD) measured at 600 nm of 0.2 and an inoculum size of 3×10^9 cells/ml. Fig. 1 shows the time course of lipase production followed at 37 °C with cell growth. The lipase activity was observed to start soon after incubation and reached the maximum (30 U/ml) at the end of the exponential phase corresponding to 30-h of cultivation (Fig. 1). No proteolytic activity was detected in the culture medium when using azocasein as substrate (data not shown).

Lipases are generally produced using carbon source such as oils, fatty acids, glycerol or tweens in the presence of an organic nitrogen source [28]. In contrast to most microbial lipases and like SSL [26] and SXL [27], the production of SAL3 is not induced by the presence of triacylglycerols (like TC_4 or olive oil) or esters (Tween 80).

3.2. Purification of lipase

The SAL3 was purified according to the procedure described in the Section 2.8. The purified lipase was homogenous when tested by coomassie blue staining of SDS-PAGE (Fig. 2, line 4). This figure shows that one band was revealed for SAL3 with a molecular mass of about 45 kDa.

To determine the molecular mass of the native enzyme, the protein elution profile obtained at the final purification step, were pooled, lyophilised and resuspended in 100 mM phosphate buffer, 150 mM NaCl, pH 7. Two hundred micrograms of the pure enzyme was loaded on size exclusion HPLC column. The lipase emerged at 5.18 min after injection corresponding to a molecular mass of 180 kDa (data not shown).

These results (SDS-PAGE and gel filtration) suggested that SAL3 was a tetrameric protein. This observation is in accordance with those obtained with some staphylococcal lipases (SAL NCTC8530, SHL, SSL), which form an aggregate [9–11].

The purification flow sheet is shown in Table 1. The specific activity of the pure lipase reached 4200 U/mg using TC_4 as substrate in the presence of 2 mM $CaCl_2$, 1 mM NaDC at pH 9.5 and 55 °C. The specific activity measured was four or two-fold higher than the one of SSL [26] or SXL [27], respectively. Under the same conditions, a specific activity of 3500 U/mg was obtained when using gum Arabic emulsified olive oil as substrate in the presence of 2 mM $CaCl_2$, 2 mM NaDC. These results show that, unlike SAL NCTC8530 which are highly selective for short-chain substrates, SAL3 hydrolyses different chain length triacylglycerol at high rates. No phospholipase activity was detected when using egg PC as substrate. Simons et

Table 1
Flow sheet of the SAL3 purification

Purification step	Total activity (U)[a]	Proteins[b] (mg)	Specific activity (U/mg)	Activity recovery (%)	Purification factor
Culture supernatant	15000	1500	10	100	1
$(NH_4)_2SO_4$ precipitation	12300	875	14	82	1.4
Heat treatment (3 min at 55 °C)	11200	710	15.7	74.6	1.57
G-100 chromatography	9000	71	127	60	12.7
Mono-S chromatography	4600	7.65	600	30.6	60
S-200 chromatography	3450	0.82	4200	23	420

[a] 1 Unit corresponds to 1 μmol of fatty acid released per minute using tributyrin as substrate.
[b] Proteins were estimated by the Bradford method [20]. Experiments were done in triplicate.

al. have shown that SHL hydrolyses both triacylglycerols and phospholipids irrespective of their chain length [14].

3.3. N-terminal sequence of SAL3

The SAL3 NH_2-terminal sequencing allowed the identification of 15 residues, L-K-A-N-Q-V-Q-P-L-N-K-Y-P-V-V. This N-terminal sequence exhibits a high degree of homology with lipases of the same genus previously characterised [26,29]. Two additional amino acid residues (LK) are present at the N-terminus of purified lipase from *S. aureus* as compared to *Staphylococcus simulans* lipase [26]. The presence of the additional dipeptide Leu-Lys at the N-terminus of the SAL3 might originate from a different proteolytic cleavage during processing of the mature enzyme.

3.4. Cloning and sequencing of the mature lipase gene region

The PCR product corresponding to the gene part encoding the mature SAL3 was cloned and sequenced. It revealed a sequence of 1191 nucleotides (Fig. 3A). The deduced polypeptide sequence, corresponding to the mature protein, comprises 397 amino acids with a molecular mass of 45 kDa. Fig. 3B shows a structure-based alignment of the SAL3 sequence with SHL, a highly homologous lipase of known 3D structure [17]. The two lipases share 52% identity in their primary sequence yet markedly differ in chain length preference and thermostability [17]. From this sequence alignment (Fig. 3B) the catalytic triad (residues Ser119, Asp310 and His352) and the calcium-binding site (residues Asp351, Asp354, Asp359, Asp362 and Gly286) of SAL3 could be identified. The amino acid sequence of SAL3 also shows 99.49% identity with the mature amino acid sequence of SSL [26] with the addition of two amino acid residues (LK) at the N-terminus. Recently, *Staphylococcus xylosus* lipase (SXL) was cloned, sequenced and expressed in *E. coli* [27,30]. The amino acid sequence of this mature lipase presents 99.7% identity with the mature amino acid sequence of SSL (GenBank accession no. AY701336) with one substitution of Gly311 by Val [30]. It has been shown that the residue G311 contributes significantly on substrate discrimination, pH and temperature dependency of SXL [31].

3.5. Interfacial activation of SAL3

The hydrolysis rate of TC_3 emulsified in 0.33% GA and 0.15 M NaCl by SAL3 as a function of substrate concentration shows a normal Michaelis–Menten dependence of the activity on the substrate concentration (Fig. 4). The interfacial activation cannot be taken as the unique criterion required to distinguish lipases from esterases [32]. Lipases are defined as a family of enzymes able to hydrolyse long-chain triacylglycerols independently of the presence, or the absence, of the interfacial activation phenomenon. Here, we can say that SAL3, which hydrolyses efficiently olive oil, is a true lipase.

3.6. Kinetic studies of SAL3

It has been established that some microbial lipases like *Rhizopus oryzae* lipase [33] may lack enzymatic activity when TC_4 is used as

(A)
```
cttaaagcgaatcaagtacaaccacttaacaaatatccagttgtttttgtacatggattt 60
  L  K  A  N  Q  V  Q  P  L  N  K  Y  P  V  V  F  V  H  G  F  20
ttaggattagtaggcgataatgcacctgctttatacccaaattattggggtggaaataaa 120
 L  G  L  V  G  D  N  A  P  A  L  Y  P  N  Y  W  G  G  N  K  40
tttaaagttatcgaagaattgagaaagcaaggctataatgtacatcaagcaagtgttagt 180
 F  K  V  I  E  E  L  R  K  Q  G  Y  N  V  H  Q  A  S  V  S  60
gcatttggtagtaactatgatcgcgctgtagaactttattattacattaaaggtggtcgc 240
 A  F  G  S  N  Y  D  R  A  V  E  L  Y  Y  Y  I  K  G  G  R  80
gtagattatggcgcagcacatgcagctaaatacggacatgagcgctatggtaagacttat 300
 V  D  Y  G  A  A  H  A  A  K  Y  G  H  E  R  Y  G  K  T  Y  100
aaaggaatcatgcctaattgggaacctggtaaaaaggtacatcttgtagggcatagtatg 360
 K  G  I  M  P  N  W  E  P  G  K  K  V  H  L  V  G  H  S  M  120
ggtggtcaaacaattcgtttaatggaagagtttttaagaaatggtaacaaagaagaaatt 420
 G  G  Q  T  I  R  L  M  E  E  F  L  R  N  G  N  K  E  E  I  140
gcctatcataaagcgcatggtggagaaatatcaccattattcactggtggtcataacaat 480
 A  Y  H  K  A  H  G  G  E  I  S  P  L  F  T  G  G  H  N  N  160
atggttgcatcaatcacaacattagcaacaccacataatggttcacaagcagctgataag 540
 M  V  A  S  I  T  T  L  A  T  P  H  N  G  S  Q  A  A  D  K  180
tttggaaatacagaagctgttagaaaaatcatgttcgctttaaatcgatttatgggtaac 600
 F  G  N  T  E  A  V  R  K  I  M  F  A  L  N  R  F  M  G  N  200
aagtattcgaatatcgatttaggattaacgcaatggggctttaaacaattaccaaatgag 660
 K  Y  S  N  I  D  L  G  L  T  Q  W  G  F  K  Q  L  P  N  E  220
agttacattgactatataaaacgcgttagtaaaagcaaaatttggacatcagacgacaat 720
 S  Y  I  D  Y  I  K  R  V  S  K  S  K  I  W  T  S  D  D  N  240
gctgcctatgatttaacgttagatggctctgcaaaattgaacaacatgacaagtatgaat 780
 A  A  Y  D  L  T  L  D  G  S  A  K  L  N  N  M  T  S  M  N  260
cctaatattacgtatacgacttatacaggtgtatcatctcatactggtccattaggttat 840
 P  N  I  T  Y  T  T  Y  T  G  V  S  S  H  T  G  P  L  G  Y  280
gaaaatcctgatttaggtacatttttcttaatggatacaacgagtagaattattggtcat 900
 E  N  P  D  L  G  T  F  F  L  M  D  T  T  S  R  I  I  G  H  300
gatgcaagagaagaatggcgtaaaaatgatggtgtcgtaccagtgatttcgtcattacat 960
 D  A  R  E  E  W  R  K  N  D  G  V  V  P  V  I  S  S  L  H  320
ccgtccaatcaaccatttgttaatgttacgaatgatgaacctgccacacgcagaggtatc 1020
 P  S  N  Q  P  F  V  N  V  T  N  D  E  P  A  T  R  R  G  I  340
tggcaagtcaaaccaatcatacaaggatgggatcatgtcgattttattggtgtggacttc 1080
 W  Q  V  K  P  I  I  Q  G  W  D  H  V  D  F  I  G  V  D  F  360
ctggatttcaaacgtaaaggtgcagaacttgccaacttctatacaggtattataaatgac 1140
 L  D  F  K  R  K  G  A  E  L  A  N  F  Y  T  G  I  I  N  D  380
ttgttgcgtgttgaagcgactgaaagtaaaggaacacaattgaaagcaagttaa       1194
 L  L  R  V  E  A  T  E  S  K  G  T  Q  L  K  A  S Stop      397
```

Fig. 3. (A) Nucleotide sequence of the SAL3 gene part encoding the mature lipase and the deduced amino acid sequence. Experiment was performed three times from three different clones. No difference was observed (see Section 2). The amino acid sequence obtained by sequencing the N-terminal of the pure SAL3 is in bold. The differences between SAL3 and SSL sequences [27] are underlined. (B) Alignment of the amino acid sequences of the mature forms of SAL3 (present study, EU035270), SSL [27], SAL NCTC8530 [29], SEL [16] and SHL [17]. Boxes in black indicate positions at which the amino acids are identical in the five proteins. Boxes in gray indicate the location of similar residues of the five protein sequences. The closed triangles indicate the Ser, Asp and His which form the catalytic triad. Stars indicate the calcium-binding residues. The dashes represent gaps introduced during the alignment process. The alignment was generated with BioEdit software.

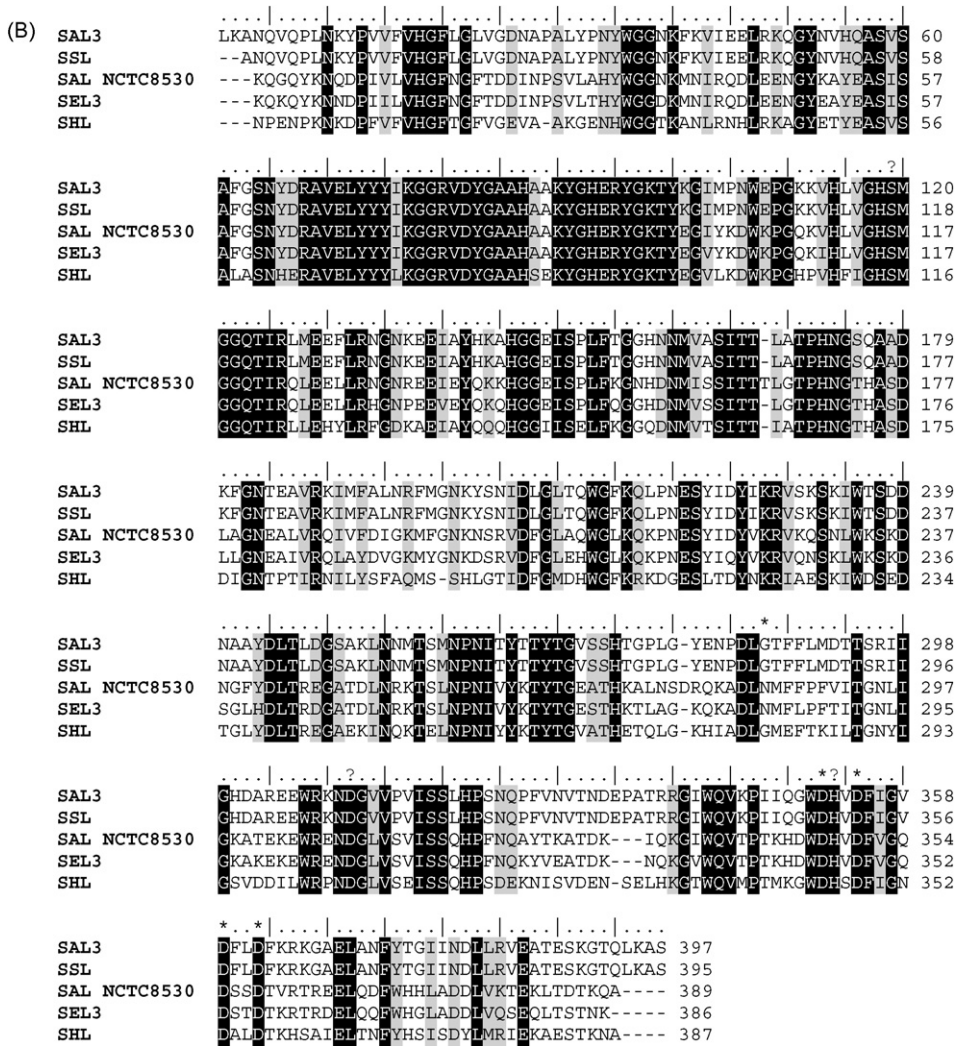

Fig. 3. (*Continued*).

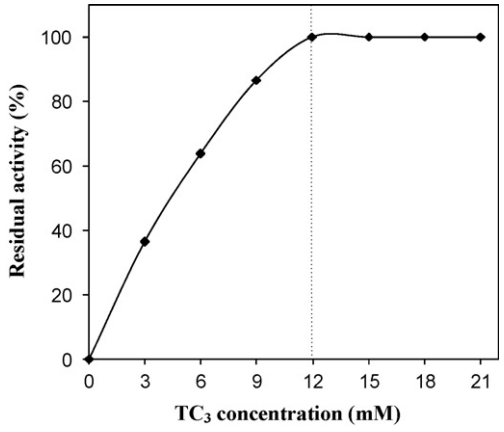

Fig. 4. Hydrolysis rate of TC_3 by SAL3 as function of substrate concentration. The TC_3 solutions were systematically prepared by mixing (3×30 s in a warring blender) a given amount of TC_3 in 30 ml of 0.3% GA and 0.15 M NaCl. The release of propionic acid was recorded continuously at pH 7 and 37 °C using a pH-stat. The CMC of TC_3 (12 mM) is indicated by vertical dotted lines.

substrate in the absence of amphipathic reagent. The high energy existing at the tributyrin/water interface is responsible for the irreversible denaturation [34]. SAL3 is able to hydrolyse the TC_4 or the olive oil emulsion alone. The kinetic remains linear for more than 15 min (data not shown). Accordingly, SAL3 probably presents a tridimentionnel structure allowing it to hydrolyse its substrate efficiently and without any denaturation at high interfacial energy (TC_4), and to tolerate the presence of long-chain free fatty acids, at the olive oil/water interface without any addition of amphipathic reagent (NaDC, Triton X-100 . . .).

Another important difference between staphylococcal lipases is their chain length selectivity. Both SAL NCTC8530 [14] and SEL [16] have a strongly preference for short-chain substrates, whereas SHL [14], SSL [26] and SXL [27] hydrolyse triacylglycerols or *p*-nitrophenyl esters almost irrespective of their chain length. The activity of SAL3 was studied using TG with varying chain length as substrates. Obviously, SAL3 activity strongly depends on the chain length of the substrate. The lipase is mainly active on short-chain triacylglcerol (TC_4) and enzymatic activity decreased when long-chain triacylglycerols were used as substrate.

The kinetic parameters of SAL3 were also studied by determining the rates of hydrolysis of different concentrations of pure TC_4, TC_8 or TC_{18}. The Lineweaver-Burk curves were plotted. From these fits, the apparent substrate affinity constants ($K_{M\,app.}$) and the max-

Table 2
Chain length selectivity and kinetic parameters of SAL3

Substrate	$K_{M\,app.}$ (mM)	V_{max} (μmol/min/mg)	k_{cat} (s^{-1})	$k_{cat}/K_{M\,app.}$ (s^{-1} mM^{-1})
TC_4	4.93	7774	5889	1194
TC_8	7.87	2803	2123	270
TC_{18}	14.53	1485	1125	77

Activity measurements were performed using pure TC_4, TC_8 or TC_{18} at pH 9.5 and 55 °C.

imum velocity values (V_{max}) of the purified lipase were obtained and shown in Table 2.

Our results showed that the $K_{M\,app.}$ value increased with increasing the acyl-chain length of the substrate. In contrast, the k_{cat} value decreased with the decrease of the aliphatic chain length of the triacylglycerols. The deduced value of the catalytic efficiency ($k_{cat}/K_{M\,app.}$) of SAL3 was 1194.6 when using TC_4 as substrate. However, 4.4 or 15.4-fold decrease in catalytic efficiency was observed when using TC_8 or TC_{18} as substrate, respectively. This result confirms that SAL3 hydrolyses more efficiently short than long-chain triacylglycerols.

3.7. Effects of pH and temperature on SAL3 activity and stability

The importance of alkaline and thermostable lipases for different applications has been growing rapidly. A great deal of research is currently going into developing lipases, which will work under alkaline conditions as fat stain removers.

The activity of SAL3 was investigated at different pH using tributyrine as substrate. Unlike all previously described staphylococcal lipases [16], our results show that SAL3 remains active at a pH range of 8–10 (Fig. 5A). Under the same experimental conditions, the SSL or SXL activity was maximal at pH 8.5 or 8.2, respectively [26,27].

In the pH stability study, the lipase is stable at abroad range of pH values between pH 5–12 after 24 h incubating (Fig. 5B). Under the same experimental conditions, the SSL or SXL was found to be stable between pH 4 and 9 or pH 5 and 8.5, respectively [26,27]. Lipases active and stable in alkaline media are very attracting, for example, lipase produced by *Acinetobacter radioresistens* has an optimum pH of 10 and it was stable over a pH range of 6–10; this enzyme has a great potential for application in the detergent industry [35].

The lipase activity was also determined at different temperatures under standard assay conditions (Fig. 5C). In contrast to SSL or SXL, which is active at 37 or 45 °C, respectively [26,27], as well as to all staphylococcal lipases described so for [16], the SAL3 activity increased significantly with increasing the temperature to reach its maximum value at 55 °C. The thermostability of SAL3 was also determined by measuring the residual activity after incubation of the pure enzyme at various temperatures (Fig. 5D). In contrast to

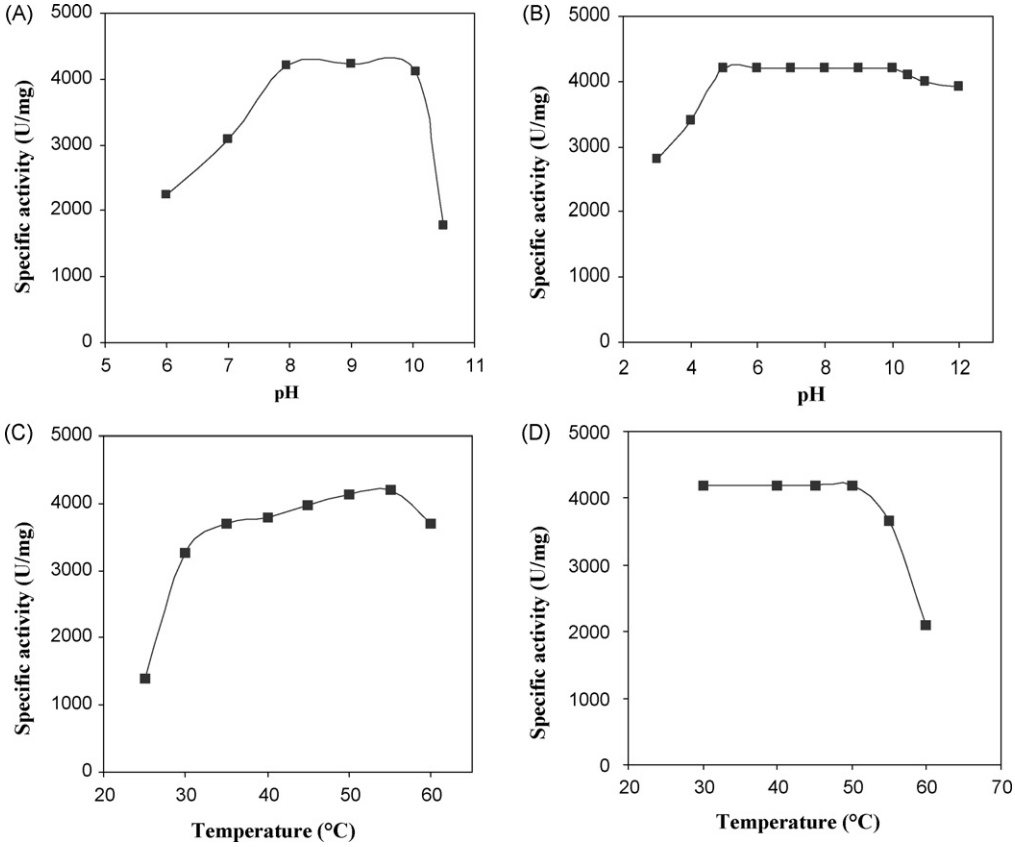

Fig. 5. pH effect on enzyme activity (A) and stability (B) of SAL3. Optimal pH was determined with tributyrin at 55 °C under the standard conditions. Stability was analysed after preincubating the pure enzyme for 24 h in different buffer solutions at various pH ranging from 3 to 12. Temperature effect of on SAL3 activity (C) and stability (D). For temperature stability the pure enzyme was preincubated at different temperatures for 1 h and the remaining activity was measured under the standard conditions.

SSL which is inactivated after a few minutes when incubated at 60 °C [26], SAL3 retained 95% or 50% of its activity after a 60 min incubation at 55 or 60 °C, respectively.

Thermostable and alkaline lipases are therefore highly attractive [36] to the synthesis of biopolymers and biodiesel and used for the production of pharmaceuticals, agrochemicals, cosmetics, and flavour [37].

3.8. Effects of calcium on SAL3 activity

Metal cations, particularly Ca^{2+}, play important roles in influencing the structure and function of enzyme, and calcium-stimulated lipases have been reported [38].

Previously, it has been demonstrated that the activity of staphylococcal lipases may depend on the presence of Ca^{2+} ions [16]. The effect of various Ca^{2+} concentrations on the rate of hydrolysis of SAL3 was studied. Our results showed that SAL3 activity can be detected in the absence of Ca^{2+}. A specific activity of 1500 U/mg was measured in the presence of 10 mM of chelator such as EDTA or EGTA when using tributyrin as substrate. In the absence of chelators, the specific activity of SAL3 reached 4200 U/mg at 2 mM $CaCl_2$ (data not shown).

The enzymatic activity of staphylococcal lipases is stimulated by Ca^{2+}. It has been reported that the lipases from *P. glumae* [39] and *S. hyicus* [16,17] contain a Ca^{2+}-binding site which is formed by two conserved aspartic acid residues near the active-site, and that binding of the Ca^{2+} ion to this site dramatically enhanced the activities of these enzymes. Both residues are conserved in SAL3 as well as in all other staphylococcal lipases sequences, indicating that these lipases probably bind Ca^{2+} at the same site [15].

3.9. Effect of detergents on lipase activity and stability

The most commercially important field of application for hydrolytic lipases is their addition to detergents, which are used mainly in household and industrial laundry and in household dishwashers.

In order to check if the purified SAL3 is able to hydrolyse triacylglycerols in the presence of surface-active agents like natural (NaDC) or synthetic (TX-100) detergent, we have measured the rate of hydrolysis of TC_4 by SAL3 in the presence of varying concentrations of NaDC or TX-100 (Fig. 6). Our results showed that the specific activity of SAL3 reached 4200 U/mg at 1 mM NaDC and that this natural detergent has no inhibitory effect on the SAL3 activity even at a large concentration (10 mM). The lipolytic activity of the enzyme was strongly amplified in presence of 4 mM TX-100 and displays a 1.6-fold increase compared to the activity in the standard condition.

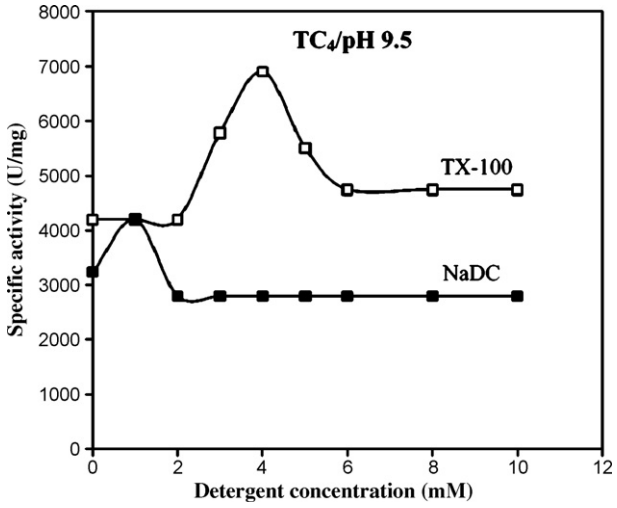

Fig. 6. Effect of increasing concentration of detergent (NaDC, TX-100) on the hydrolysis rate of tributyrine by SAL3. Lipolytic activity was measured at pH 9.5 and 55 °C using a pH-stat.

Table 3
Lipase stability of SAL3, SXL or SSL in presence of surfactants, detergents and oxidizing agents

Surfactants/detergents/oxidizing agents	SAL3	SXL	SSL
Control	100	100	100
Surfactants			
Triton X-100	100	83	33
Tween-80	100	95	58
Sodium cholate	100	41	50
Sodium taurocholate	100	37	33
Detergents			
Ariel	100	38	25
Axion	100	61	8
Omino Bianco	100	38	41
Oxidizing agents			
Hydrogen peroxide	60	34	0
Sodium perborate	70	53	0

SAL3, SXL or SSL preparation was incubated in presence of surfactants (20 mM), commercial detergents (1%, w/v) and oxidizing agents (10%, v/v) for 1 h at 40 °C. Residual SAL3 activity was determined at pH 9.5 and 55 °C. Residual SSL or SXL activity was measured under the standard conditions of each enzyme as described previously [26,27]. All experiments were done in triplicate.

These results confirm that, in contrast to many lipases described so far from different origins [33,40], the presence of some surface-active agents like bile salt or TX-100, does not affect the behaviour of the enzyme towards TC_4–water interfaces.

Besides, the present lipase exhibited temperature and pH suitable for detergent formulation, the compatibility of SAL3 in the presence of some surfactants was checked. Results presented in Table 3 reveal that SAL3 is highly stable towards some known surfactants and retained its full activity in the presence of 20 mM Triton TX-100, NaDC, NaTDC and Tween 80 (Table 3).

The compatibility of SAL3 in the presence of some commercial detergents was also studied. As we can see from Table 3, SAL3 retained 100% of its maximum activity in the presence of Axion, Ariel or Omino Bianco. To allow further comparison we report in the same Table 3 the results obtained by SSL and SXL. One can say that SAL3 represents the most resistance towards commercial detergents as compared to other staphylococcal lipases (SSL or SXL).

Bleach stability of the enzyme was also checked in the presence of hydrogen peroxide and sodium perborate (Table 3). Remarkably, this novel enzyme exhibited, in contrast to SSL or SXL, high stability towards these two agents. In fact, 60% or 70% of residual lipase activity was measured after 1 h incubation at 40 °C in the presence of 10% hydrogen peroxide or sodium perborate, respectively.

Considering the overall properties of different staphylococcal lipases and the one described in this study, one can say that SAL3 can be considered as a potential candidate to be used as in biotechnology and essentially for application in the detergent industry.

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