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The N-terminal His-tag and the recombination process affect the biochemical properties of *Staphylococcus aureus* lipase produced in *Escherichia coli*

Habib Horchani, Selmene Ouertani, Youssef Gargouri, Adel Sayari*

Laboratoire de Biochimie et de Génie Enzymatique des Lipases, ENIS, BPW 3038 Sfax, Tunisia

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ABSTRACT

The part of the gene encoding the mature *Staphylococcus aureus* lipase (SAL3) was cloned using PCR technique. The sequence corresponding to the mature lipase was subcloned into pET-14b or pOP-T expression vector and expressed in *E. coli* BL21 (DE3). The tagged (His_6-SAL3) and untagged (r-SAL3) *Staphylococcus aureus* lipases were purified to homogeneity using Ni-NTA resin and classical chromatographic techniques, respectively. We performed a comparative study on the biochemical properties of two recombinant *Staphylococcus aureus* lipases and the wild type form. The major differences among these lipases are mainly reflected in their stability at high and low temperature, measured in aqueous media as well as in various organic solvents. Furthermore, our results indicate that the presence of the His-tag in the N-terminus of the SAL3 as well as the recombination process significantly affect the adsorption of these proteins onto $CaCO_3$ used as support and their capacity to synthesise diesel additive by esterification of butanol with oleic acid.

1. Introduction

Lipases (glycerol ester hydrolases, EC 3.1.1.3) catalyse the hydrolysis of triglycerides and several other substrates containing ester bonds [1,2] with an astonishing variety in their regio- and stereospecificity as well in their selectivity towards the length and saturation degree of the acyl chain. They are widely produced by microorganisms, plants, and animals. Microbial lipases have attracted considerable attention due to their biotechnological potential ranging from their use as additives in laundry detergents, textiles or foods to stereospecific biocatalysts [3].

The production of extracellular lipase by staphylococcal species has been known for many years [4]. Interest in these enzymes was originally stimulated by observations that certain pathogenical staphylococci possess lipolytic activity. Jaeger et al. [5] established that microbial lipases, like that of the *Staphylococcus aureus*, may be involved in pathological related to lipid hydrolysis [5]. In addition to this physiological importance, research was stimulated by potential use of staphylococcal lipases in industry.

In our laboratory, *Staphylococcus simulans* lipase (SSL) [6] and *Staphylococcus xylosus* lipase (SXL) [7] were purified. These lipases were used to synthesise monoolein, which is the most widely used emulsifier in food and pharmaceutical industries [8], and aromatic molecules (ethyl valerate and hexyl acetate) [9]. In addition, it has been previously reported that the SXL is an important catalyst to synthesize acetylated tyrosol which has an antioxidant effect [10].

In a previous study, a lipase from *Staphylococcus aureus* (wt-SAL3) was purified and characterized. It has attracted more attention since it has the potential to be used in detergent industries due to the stability of this enzyme at high temperature, alkaline conditions and in many detergents [11]. However, the expression efficiency of this lipase in wild-type *S. aureus* is low and this has limited its broad utilisation. Expression of foreign protein in prokaryotic systems is the most widely used approach to achieve high level expression for both fundamental studies and commercial purposes. In addition, the expression vectors and hosts are critical issues for achieving maximal expression. Therefore, several cloning vectors containing a C- or N-terminal in-frame sequence for His-tag have been engineered to enable the expression of recombinant proteins in prokaryotic and eukaryotic expression systems [12–16].

For large-scale purification of recombinant proteins, the use of affinity tags is usually preferred due to the specificity of the process and the relatively easy purification schemes. Although, universally applicable, the use of the His-tag and the affinity chromatography

Abbreviations: SAL3, *Staphylococcus aureus* lipase; r-SAL3, recombinant untagged *Staphylococcus aureus* lipase; His_6-SAL3, recombinant tagged *Staphylococcus aureus* lipase; SSL, *Staphylococcus simulans* lipase; SXL, *Staphylococcus xylosus* lipase; SAL NCTC8530, *Staphylococcus aureus* NCTC8530 lipase; SHL, *Staphylococcus hyicus* lipase; BSA, bovine serum albumin; EDTA, ethylene diamine tetraacetic acid; NaDC, sodium deoxycholate; NaTDC, sodium taurodeoxycholate; PC, phosphatidylcholine; PCR, polymerase chain reaction; SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; TG, triacylglycerol; TC_4, tributyrin; TC_8, trioctanoine; OO, olive oil.

* Corresponding author. Tel.: +216 74 675055; fax: +216 74 675055.
E-mail address: adelsayari@yahoo.fr (A. Sayari).

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is not recommended for proteins containing metal ions. Additionally, introducing an affinity tag has also been reported to negatively affect the target protein resulting in a change in protein conformation [17], undesired flexibility in structure studies [18], inhibition of enzyme activity, toxicity [19,20] and alteration in biological activity.

The aim of this study is to evaluate the influence of the N-terminal Histidine tag extension and the importance of the recombination process on the biochemical properties of recombinant *Staphylococcus aureus* lipase. Two recombinant lipases were expressed in *E. coli*, an untagged SAL3 lipase named (r-SAL3), identical to the wild type lipase (wt-SAL3) (without any extension at the N-terminal) and an Histidine tagged lipase named (His_6-SAL3), identical to wt-SAL3 but carrying the N-terminal tag (M-R-G-S-H-H-H-H-H-H-S-S-G-L-V-P-R-G-S). Expression levels and some biochemical properties of the two recombinant lipases were determined and compared to the wild type one.

2. Experimental

2.1. Chemicals

Isopropyl thio-β-D-galactopyranoside (IPTG) was purchased from Boehringer. Tributyrin (99%; puriss) and benzamidine were from Fluka (Buchs, Switzerland), deoxycholic acid (NaDC), yeast extract and ethylene diamine tetraacetic acid (EDTA) were from Sigma Chemical (St. Louis, USA). Yeast extract, phosphatidylcholine were from Sigma Chemical (St. Louis, USA). Marker proteins and supports of chromatography used for lipase purification: Sephadex G-100, Mono S-Sepharose and Mono Q-Sepharose gels were from Pharmacia (Uppsala, Sweden); PVDF membrane was purchased from Applied Biosystems (Roissy, France). Oligonucleotides were synthesised by GENOME Express (Grenoble, France).

2.2. Bacterial strains, plasmids, and vectors

Escherichia coli strain DH5α was used as cloning host. For expression, *E. coli* BL21 (DE3) strain, which contains the structural gene for T7 RNA polymerase under control of the *lac* promoter, was used. *E. coli* strains were grown in Luria–Bertani medium, supplemented with 100 μg/ml ampicillin when plasmid maintenance was required. The plasmid pCR® 4Blunt-TOPO® (Invitrogen Corporation) was used as cloning vector. The plasmid pET-14b (Invitrogen) and pOP-T (given by olga Pericic, MRC Center, Cambridge, UK) under the control of the T7 promoter, were used for the over expression of the part of the gene encoding the mature SAL3. PCR products were purified using Wizard PCR Preps DNA Purification System (Promega).

2.3. DNA preparation and transformation procedure

Staphylococcal DNA was prepared as described previously [11]. Cells were lysed by the addition of lysostaphin (sigma) at 4.5 U/ml. *E. coli* supercoiled plasmid DNA was prepared by the modified alkaline lysis method [21]. *E. coli* was transformed by the $CaCl_2$ method. Enzymes for molecular cloning were obtained from Promega. Assay conditions were in agreement with suppliers' recommendations.

2.4. Cloning of the mature lipase gene region

The part of the gene encoding the mature lipase was amplified by PCR as described previously from genomic DNA of *S. aureus* [11]. Forward primer was 5′-GATCGAATTCATATGTTAAAA-GCGAATCAAGTACAACCACT-3′ and reverse primer was 5′-GATCGAATTCGGATCCTTAACTTGCTTTCAATTGTGTT-3′. The PCR product (1.2 kb) was isolated and ligated into the EcoRI-linearised and dephosphorylated pCR® 4Blunt-TOPO®, using the pCR® 4Blunt-TOPO® according to manufacturer's protocol. 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 restriction analysis. DNA products were analysed on a standard 1% agarose gel containing ethidium bromide. DNA sequences were elucidated by 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) as template with T7 promoter primer and the T3 primer (Amersham Pharmacia Biotech).

2.5. Construction of over expression plasmids

The recombinant vector (pSAL3) was double digested by NdeI/BamHI. The double digestion product was purified, and ligated into a previously NdeI/BamHI linearised and dephosphorylated pOP-T and pET-14b vectors. This construction places the lipase gene under control of a T7 promoter, which greatly accelerated the protein purification process. The constructed pOP-T or pET-14b-SAL3 plasmids were transformed into *E. coli* DH5α competent cells. The positive colonies with a gene insert in the plasmids were identified by double digestion of the plasmids with NdeI/BamHI, followed by agarose gel analysis. The identified positive colonies were grown in LB medium containing ampicillin (100 μg/ml), and the plasmids pOP-T-SAL3 and pET-14b-SAL3 were isolated from bacteria cells using a plasmid extraction kit (Promega). The isolated pET-14b-SAL3 and pOP-T-SAL3 plasmids were then used to transform *E. coli* strain BL21 (DE3) competent cells for expression purposes.

2.6. Comparison of expression levels between the His_6-SAL3 and the r-SAL3

The *E. coli* strain BL21 (DE3) cells harbouring pET-14b-SAL3 or pOP-T-SAL3 plasmids were grown at 37 °C in 50 ml LB medium containing 100 μg/ml ampicillin to an OD_{600} of 0.2. The culture of each enzyme was then adjusted to 0.4 mM IPTG and incubation continued at 37 °C for 24 h. During the cultivation 1 ml samples were taken to measure the lipase activity and the OD_{600}. After 14 h of incubation time, cells were harvested by centrifugation at 6000 rpm for 10 min and washed twice with buffer A (20 mM Tris–HCl, pH 8, 50 mM NaCl, 2 mM benzamidine). The culture broth obtained was collected and assayed for secreted or extracellular lipase activity. The cells, collected by centrifugation, were resuspended in the buffer A, and sonicated with a 130-W autotune series High Intensity Ultrasonic sonicator, equipped with 6 mm diameter tip, to release intracellular proteins. The cell-free extract was centrifuged at 10,000 rpm for 20 min to remove cell debris and assayed for lipase activity. Thereafter, the two recombinant lipases (tagged and untagged) are purified with two different chromatographic protocols.

2.7. Purification of the His_6-SAL3

As mentioned above, after the last centrifugation, the clear supernatant obtained was mixed with 5 ml of the Ni^{2+} nitrilotriacetate (NTA) resin (Qiagen, CA, USA) equilibrated with buffer A. The crude extract–NTA mixture was loaded into a chromatographic column and washed with 200 ml buffer A. Lipases were eluted with a linear imidazole gradient (100 ml of 0–300 mM in buffer A).

2.8. Purification of the r-SAL3

The clear supernatant, obtained after sonication, containing lipase was used as the crude enzyme preparation for the following

chromatographic steps.

- *Filtration on Sephadex G-100*. The enzyme solution (5 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 were pooled.
- *Anion exchange chromatography*. Active fractions eluted from Sephadex G-100 column were poured into a Mono Q-Sepharose anion 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. Lipase activity was eluted between 150 and 300 mM NaCl.
- The fractions containing the lipase activity were pooled, concentrated, diluted in buffer B (20 mM sodium acetate pH 5.4, 50 mM NaCl, and 2 mM benzamidine) and loaded on cation exchange chromatography (2 cm × 30 cm) equilibrated in the same buffer. Elution was performed with a linear gradient (300 ml of 20–500 mM in buffer B) at a rate of 45 ml/h. lipase activity was eluted between 200 and 400 mM NaCl.

2.9. Lipase and phospholipase activities determination

The lipase activity was measured by continuous and automated titration of liberated fatty acids using a pH-stat. Activities were measured under standard conditions using tributyrin (0.25 ml) in 30 ml of 2.5 mM Tris–HCl, 2 mM $CaCl_2$, and 1 mM NaDC or olive oil emulsion (10 ml in 20 ml of 2.5 mM Tris–HCl pH 8.5, 2 mM $CaCl_2$ and 2 mM NaDC) as substrate.

The phospholipase activity was checked titrimetrically under the standard conditions of each enzyme 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.

The catalytic constants (k_{cat}) values were calculated from the maximal velocities (V_{max}) values recorded in presence of an excess of substrate concentration, using mass of 45 kDa or 43 kDa for the His_6-SAL3 or the r-SAL3, respectively. The apparent Michaelis constant ($K_{M\,app.}$) values were determined graphically from double reciprocal Lineweaver–Burk plots of the kinetic data. Lipolytic activity was expressed as units. One unit corresponds to 1 μmol of fatty acid released per minute.

2.10. Analytical methods

Analytical polyacrylamide gel electrophoresis of proteins in the presence of sodium dodecyl sulphate (SDS-PAGE) was performed following the method of Laemmli [22]. Samples for sequencing are electroblotted according to Bergman and Jörnvall [23]. Protein transfer was performed during 1 h at 1 mA/cm^2 at room temperature.

2.11. Amino acid sequencing

The N-terminal sequence of r-SAL3 was kindly determined by Pr. Hafedh MEJDOUB with automated Edaman's degradation, using an Applied Biosystems Protein Sequencer Procise 492 CLC.

2.12. Effects of pH and temperature on enzyme activity and stability

- The activity of the recombinant lipases was tested at different pH (5–10.5) at 55 °C. The pH stability of the lipases was checked by incubating each enzyme at different pH (3–12) for 24 h at room temperature. The residual lipase activity was measured, after centrifugation, under standard assay method.
- The optimal temperature for the His_6-SAL3 or the r-SAL3 activity was determined by carrying out the enzyme at different temperatures (25–60 °C). The effect of temperature on lipase stability was determined by incubating each enzyme solution at various temperatures (30–60 °C) for 60 min. The residual lipase activity was determined, after centrifugation, under standard conditions. To determine the half life of each lipase, the enzyme was incubated at 50 °C, and the activity was determined periodically up to 5 h.

2.13. Storage stability of the His_6-SAL3, the r-SAL3 and the wt-SAL3

Storage stability of the wild type or the recombinant lipases at 0.1 mg/ml in buffer B was followed during 4 h at 0 and −20 °C.

2.14. Immobilization of lipases

The enzyme immobilization was made onto $CaCO_3$ as previously described [24]. At the end of the cultivation and after the sonification step, the crude lipase preparation (1500 IU of each lipase) was immobilized by simple adsorption technique into $CaCO_3$ support. The mixture was incubated 1 h at 4 °C under mild agitation. Afterwards, 10 ml of chilled acetone was added, and the suspension was filtered through a Buchner funnel. The immobilized lipase was washed again two times with 10 ml of chilled acetone, dried in a vacuum desiccator at room temperature for 6 h and stored at 4 °C until use. The yield of immobilized lipase activity was defined as the ratio of the adsorbed activity recovered at the end of the immobilization period divided by the total soluble lipase initially added to 1 g of support.

2.15. Stability of free and immobilized His_6-SAL3, r-SAL3 and wt-SAL3 in organic solvents

The stability of lipases (wt-SAL3, r-SAL3 and His_6-SAL3) in organic solvents was determined by mixing free or immobilized lipases in different solvents (1:1, v/v; 1:1, w/v) for 24 h of incubation time. After vortex, the mixture was incubated at room temperature with constant shaking in screw-capped tubes. Samples were withdrawn periodically to determine the residual activity using tributyrin as substrate under standard conditions.

2.16. Esterification reaction

The esterification reaction was performed out in screw-capped flasks. The reaction mixture containing different substrate concentrations dissolved in *n*-hexane with a working volume of 4 ml was carried out at 37 °C in the presence of 100 IU of each lipase with shaking (200 rpm). Aliquots of the reaction mixture were withdrawn periodically. The residual acid content was assayed by titration with sodium hydroxide 3.5 g/l using phenolphthalein as indicator and 2 ml of ethanol as quenching agent. The conversion percentage in ester synthesis was based on the amount of acid consumed [25]. All experiments were carried out in triplicates.

3. Results and discussion

3.1. Expression of the His_6-SAL3 and the r-SAL3

The *E. coli* BL21 (DE3) cells containing the recombinant plasmids were grown at 37 °C in 50 ml LB medium containing 100 μg/ml ampicillin to an OD_{600} of 0.2. IPTG induction of lipase expression was carried out at different times after oniculation, and the cell growth was monitored by measuring OD_{600}. It was found that the

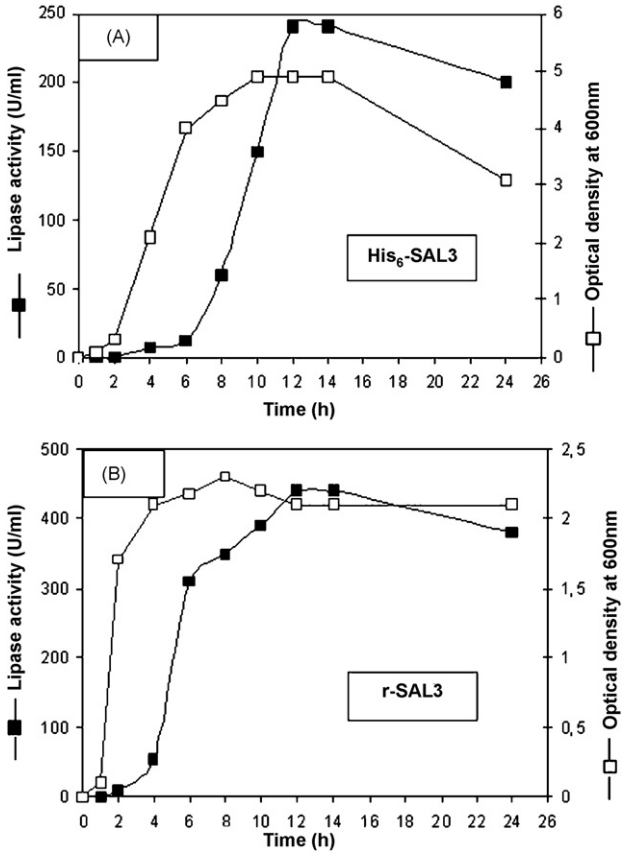

Fig. 1. The time courses of the His_6-SAL3 (A) or the r-SAL3 (B) production with cell growth of *E. coli* BL21 (DE3). The culture was carried out at 37 °C in 250 ml shaking 50 ml of LBA medium containing: 10 g/l tryptone, 10 g/l NaCl, 5 g/l yeast extract, pH 7.2 and 100 μg/ml ampicillin. Cell growth was monitored by measuring the absorbance at 600 nm. Lipolytic activity was measured using tributyrin as substrate at pH 8.5 under standard conditions.

protein expressed better at 0.4 mM IPTG concentrations and at 37 °C (data not shown). Fig. 1(A and B) shows the time course of the His_6-SAL3 and the r-SAL3 productions followed at 37 °C with cell growth. Cultivating the cells for approximately 14 h resulted in a cell density that was higher for the cells expressing the histidine tagged lipase (OD_{600} of 4.8) compared to cells expressing the untagged one (OD_{600} of 2.2). The positive effect of the histidine tag on the higher cells density could be explained by the hydrophilic nature of the histidine tag, which can increase the hydrophilic character of the tagged lipase. The last form of the enzyme could be more compatible with the host cell. Similarly, Svensson et al. (2006) reported that after cultivating the cells for approximately 24 h, the cell density was 40 and 50% higher for the cells expressing Histidine tagged amelogenin compared to cells expressing the untagged amelogenin [26].

The lipase activity of the His_6-SAL3 or the r-SAL3 was carried out on crude samples after sonification of the collected cells. At a first glance, one notices in the crude sample, an increase of lipase activity of the new constructed lipases compared to wild type one.

The lipase activity of the His_6-SAL3 was detected to start soon after incubation, and reaches the maximal (240 U/ml) at the end of the exponential phase corresponding to 14 h cultivation after IPTG induction (Fig. 1A). Whereas, under the same experimental conditions, the maximal activity of the r-SAL3 reaches 440 U/ml (Fig. 1B) at the end of the exponential phase corresponding to 14 h cultivation after IPTG induction. No extracellular lipase activity was detected in the culture medium in the two cases. We have shown previously that the production of lipase with *S. aureus* (wild type

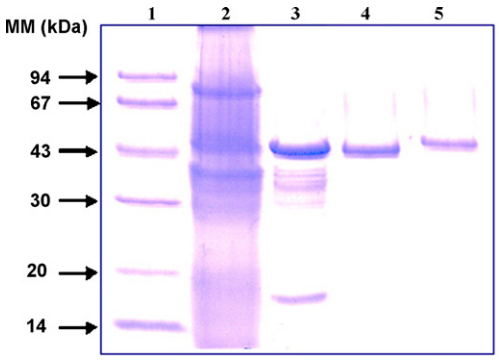

Fig. 2. SDS/PAGE (12%) analysis of the proteins purification. Lane 1, molecular mass markers; lane 2, profile of the r-SAL3 (10 μg) obtained after Sephadex G-100 chromatography; lane 3, profile of the r-SAL3 (20 μg) obtained after Mono-Q chromatography; lane 4, 10 μg of the purified r-SAL3 obtained after Mono-S chromatography; lane 5, 10 μg of the purified His_6-SAL3 obtained after Ni-NTA affinity chromatography.

SAL3) reached the maximum (30 U/ml) at the end of the exponential phase corresponding to 30 h of cultivation time with an OD_{600} of 35 [11]. Therefore, according to theses results, one can conclude that, since a decrease in the cell density for the cells expressing the recombinant lipases compared to *S. aureus*, the lipase production was improved 8 or 15 times when using pEt-14-b or pOP-T vector, respectively, compared to the wild type SAL3.

3.2. Purification of the His_6-SAL3 and the r-SAL3

- Due to the presence of hexahistidine in the construct of the His_6-SAL3, the fusion protein was purified using Ni^{2+} nitrilotriacetate (NTA) affinity column equilibrated with buffer A and eluted by a linear gradient of imidazol. This indicates that the Histidine tag is well exposed for interaction with the metal ion Ni^{2+}. This allowed us to use only one step to purify the Histidine tagged lipase using Ni-NTA affinity chromatography. After purification, the yield of lipase activity exceeds 70% (Table 1).
- The r-SAL3 was purified according to the procedure described in the Experimental section with a purification factor of about 59 (Table 1).

The results of SDS-PAGE analysis of pooled fractions of the Mono-S sepharose chromatography and the Ni-NTA affinity corresponding to the His_6-SAL3 and the r-SAL3, respectively, are shown in Fig. 2. The His_6-SAL3 or r-SAL3 exhibits a single band corresponding to a molecular mass of about 45 or 43 kDa, respectively.

The N-terminal amino acid sequence of the r-SAL3 was analysed. Edman degradation of purified lipases revealed a N-terminal sequence starting with L-K-A-N-Q-V-Q-P-L-N-K-Y. This is identical to the sequence of the wild type SAL3 previously published [11].

The purification flow sheet, given in Table 1, shows that the pure His_6-SAL3 or r-SAL3 presents a specific activity of 4000 or 4500 U/mg, respectively, when using TC_4 as substrate in presence of 2 mM $CaCl_2$ and 1 mM NaDC at the optimal temperature of each lipase.

The two enzymes are more active on short chain triacylglycerols than on long chain ones. The specific activities of the His_6-SAL3 and the r-SAL3 were 1300 or 1100 U/mg, respectively, when using olive oil emulsion as substrate under standard conditions. Moreover, the wild type SAL3 displays a specific activity of 3500 U/mg when using the same substrate.

No phospholipase activity was detected for the three proteins when egg PC was used as substrate.

Table 1
Flow sheet of the His_6-SAL3 and the r-SAL3 purification.

Enzyme	Purification step	Total activity (Units)[a]	Proteins[b] (mg)	Specific activity (U/mg)	Activity recovery (%)	Purification factor
His_6-SAL3	Culture supernatant	12,000	160	75	100	1
	Ni-NTA chromatography	8,400	2.1	4,000	70	53.3
r-SAL3	Culture supernatant	22,000	288.7	76	100	1
	G-100 chromatography	9,500	38	250	43	3.3
	Mono-Q chromatography	6,200	6.2	1,000	28	13.5
	Mono-S chromatography	4,800	1.06	4,500	22	59.2

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

3.3. Chain length selectivity of the His_6-SAL3 and the r-SAL3

The substrate chemospecificity is a criterion often used to differentiate between various lipases. *Staphylococcus aureus* NCTC 8530 lipase has been reported to hydrolyse only short chain emulsified triglycerides [27], whereas SSL [6] and SXL [7] hydrolyse both tributyrin and olive oil irrespective of their chain length. The specific activities of the two recombinant lipases (His_6-SAL3 or r-SAL3) were measured using short (tributyrin), medium (trioctanoin) or long chain triacylglycerols (olive oil) as substrate. Our results show that, like the wt-SAL3, the r-SAL3 and the His_6-SAL3 are more active on a short-chain triacylglycerol (TC_4) and the enzymatic activity is lower on a long-chain substrate (OO).

It can be seen that, when olive oil is used as a substrate, the specific activity of the recombinant tagged lipase (1300 U/mg) and that of the recombinant untagged form (1100 U/mg) are lower than that of the wild type form (3500 U/mg).

In contrast, with a partly water soluble substrate (TC_4) these above mentioned negative effects are minimum. Thus, with respect to chain length selectivity, the wt-SAL3, the r-SAL3 and the His_6-SAL3 display different behaviors. Previously, Sayari et al. (2007) have studied the chain length selectivity of the His_6-SXL and the wt-SXL. The present results confirm those obtained by Sayari et al. [28] showing that the N-terminus His-tag affects the chain length selectivity of the SXL.

To determine the kinetic parameters of lipases, the rates of hydrolysis of different concentrations of TC_4 or TC_8 were measured using the His_6-SAL3 or the r-SAL3. The Lineweaver–Burk curves were plotted (data not shown). From these fits, the apparent K_M ($K_{M\,app.}$) and the turnover of the enzymatic reaction (k_{cat}) were obtained and shown with the deduced catalytic efficiency ($k_{cat}/K_{M\,app.}$) in Table 2. Our results show that, like wt-SAL3, the catalytic efficiency value of the His_6-SAL3 and the r-SAL3 decreased with the increase of the aliphatic chain length of the triacylglycerols.

Table 2 shows that the His_6-SAL3 displays a 1.35-fold decrease in $k_{cat}/K_{M\,app.}$ values compared to r-SAL3, when using TC_4 as substrate. This decrease in the catalytic efficiency is mainly due to an increase in $K_{M\,app.}$ and the decrease of k_{cat} values. When using TC_8 as substrate, the His_6-SAL3 displays a 1.52-fold increase in $k_{cat}/K_{M\,app.}$ values compared to r-SAL3. These results show that the apparent K_M and the turnover of the enzymatic reaction (k_{cat}) are differentially affected by the addition of the His-tag to the SAL3 depending on the substrate, confirming that the His-tag in the N-terminus slightly affects the catalytic efficiency.

3.4. Effects of pH and temperature on His_6-SAL3 and r-SAL3 activity and stability

The activity of the His_6-SAL3 and r-SAL3 was investigated at different pH using tributyrin as substrate. Our results show that the two recombinant enzymes have the maximal activity at pH 8.5 (data not shown). Under the same experimental conditions, the wt-SAL3 activity was maximal at pH range of 8–10 [11]. In the pH stability, like the wt-SAL3, the His_6-SAL3 and the r-SAL3 are stable at a broad range of pH values between pH 5 and 12 after 24-h incubating (data not shown).

The optimal temperature of lipases activity was also investigated, at pH 8.5, using tributyrin as substrate (Fig. 3A). In contrast with the His_6-SAL3 and like the wt-SAL3, the r-SAL3 showed maximum activity at 55 °C (Fig. 3A). From 45 °C to 55 °C, the His_6-SAL3 activity decreased from 100% to 40%.

The thermostability of the r-SAL3 and the His_6-SAL3 was also determined by measuring the residual activity after 60-min incubation of the pure enzymes at various temperatures (data not shown). In contrast to the wt-SAL3 which retained 95% of its activity after 60-min incubation at 55 °C [11], the r-SAL3 retained 50% of its activity at this temperature. In the same conditions, the His_6-SAL3 was inactivated after a few minutes of incubation.

Our results show also that the half life value of the tagged lipase is lower than the one of the untagged lipase which is in turn lower than that of the wild type form. In fact, the His_6-SAL3 and the r-SAL3 activity was reduced to 50% after 30 min and 155 min of incubation time, respectively. In contrast, under the same conditions, the wt-SAL3 shows a half life of 330 min at 50 °C (Fig. 3B).

The difference in the thermostability between the three enzymes can be related to the enzyme refolding. The wild type lipase was probably more correctly refolded than the recombinant one. The presence of the His-tag can disturb the natural refolding. Similar results were obtained by Quyen et al. (2005) who reported that the wild type lipase from *Ralstania* sp. M1 was much more stable than the recombinant one [29].

3.5. Storage stability of the His_6-SAL3, the r-SAL3 and the wt-SAL3

In order to use identical starting material throughout the experiments, the storage stability of the pure His6-SAL3, r-SAL3 and wt-SAL3 was determined at 0 and −20 °C. After 4 h, the residual activity was determined under standard condition of each enzyme. As can be seen in Fig. 4(A and B), in contrast with the wt-SAL3, which is fully active after 4 h of storage at 0 or −20 °C, the r-SAL3 Exhibits 75 or 91% of its initial activity, respectively. Under the same conditions, the His_6-SAL3 is fully inactivated after only 3 h of incubation time. Therefore, the use of additives should be considered as an

Table 2
Kinetic parameters of the wt-SAL3, the r-SAL3 and the His_6-SAL3.

Substrate	Enzyme	$K_{M\,app.}$ (mM)	k_{cat} (s^{-1})	$k_{cat}/K_{M\,app.}$ (s^{-1} mM^{-1})	References
TC_4	wt-SAL3	4.93	5889	1194	Horchani et al. [11]
	r-SAL3	1.20	3125	2580	This work
	His_6-SAL3	1.35	2570	1905	
TC_8	wt-SAL3	7.87	2123	270	Horchani et al. [11]
	r-SAL3	13.33	2052	154	This work
	His_6-SAL3	11.75	2765	235	

Activity measurements were performed using pure TC_4 or TC_8 at standard conditions.

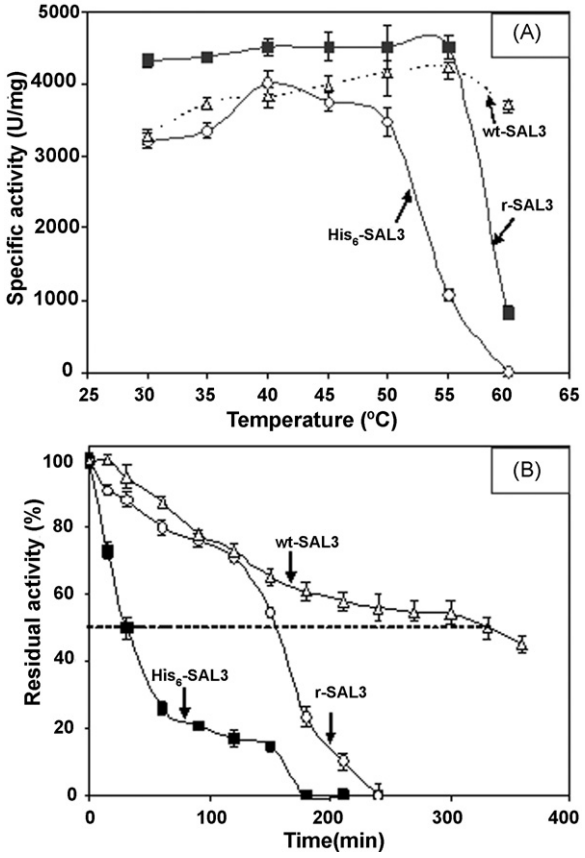

Fig. 3. (A) Temperature effect on the His_6-SAL3, the r-SAL3 and the wt-SAL3 activities. (B) Thermal inactivation of the His_6-SAL3, the r-SAL3 and the wt-SAL3 at 50 °C. Residual activities were measured with pH-stat method using tributyrin as substrate. The temperature effect on the wt-SAL3 activity, reproduced from Horchani et al. [11], is presented in dotted lines. Experiments were carried out in triplicates. The S.D. was <5%.

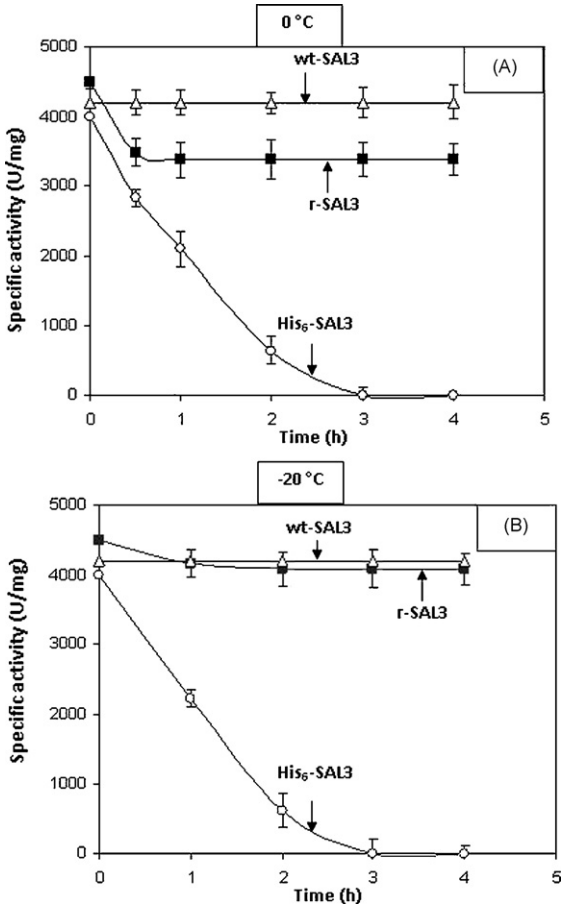

Fig. 4. Storage stability of the His_6-SAL3, the r-SAL3 and the wt-SAL3. Aliquots were stored at the indicated temperature and the residual lipase activity was measured with tributyrin as substrate under standard conditions of each enzyme. Experiments were carried out in triplicates. The S.D. was <5%.

efficient method of the His_6-SAL3 preservation. One can conclude that the His-tag significantly affects the stability of the recombinant lipase at low temperature.

3.6. Immobilization of lipases

To determine the best conditions of lipase immobilization, we studied the possibility of the wt-SAL3, the His_6-SAL3 and the r-SAL3 to be adsorbed to 1 g of $CaCO_3$ used as support. After different incubation times of an enzymatic solution (1500 IU) with 1 g of support at 4 °C, the immobilization yield was measured. As a result, a 60 min incubation time of the enzymatic solution with the support was considered ideal to reach the maximum adsorption of the three enzymes onto $CaCO_3$. The immobilization yields of the three lipases are presented in Fig. 5. As can be seen, the wt-SAL3 has been successfully immobilized onto $CaCO_3$. A good yield of immibilization (79%) was obtained after 1 h of incubation time which corresponds to the adsorption of about 1200 IU of lipases into 1 g of $CaCO_3$ used as support. In the same experimental conditions, the percentage of immobilization of the r-SAL3 or the His_6-SAL3 was 22 or 2.5%, respectively. This result indicates that the presence of the His-tag in the N-terminus of the SAL3 as well as the recombination process significantly affect the adsorption of these proteins onto $CaCO_3$ used as support. After immobilization, the wt-SAL3, the r-SAL3 and the His_6-SAL3 are fully stable at low temperature (0 or −20 °C).

3.7. Stability of free and immobilized lipases in organic solvents

Stability against organic solvents is very important when using enzymes for synthesis of esters. The stability of free or immobilized *Staphylococcus aureus* lipases in the presence of some organic solvents was studied.

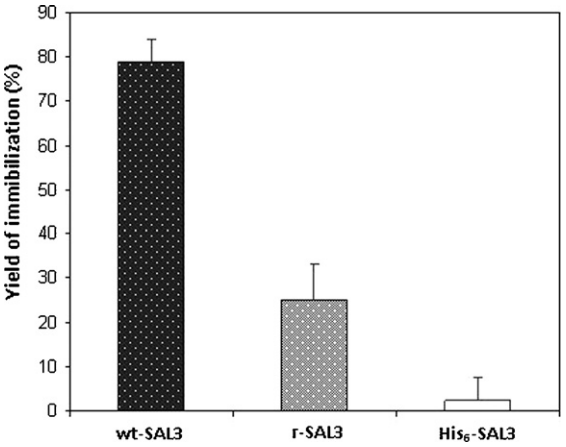

Fig. 5. Immobilization yield of the His_6-SAL3, the r-SAL3 and the wt-SAL3. Reactions were carried out using 1 g of support, 1500 IU of each lipase stirred at 4 °C during 1 h of contact. Experiments were carried out in triplicates. The S.D. was <5%.

Table 3
Stability of free or immobilized lipases in organic solvents.

	Free lipases			Immobilized lipases		
	wt-SAL3	r-SAL3	His_6-SAL3	wt-SAL3i	r-SAL3i	His_6-SAL3i
Hexane	100	100	70	100	100	80
Tert-butanol	100	0	0	100	100	70
Chloroform	100	0	0	100	82	55

As we can see in Table 3, the immobilized lipases exhibited better tolerance to organic solvents such as hexane, tert-butanol and chloroform than the free ones. In contrast to the free r-SAL3 and His_6-SAL3 which are fully inactivated after few minutes, the immobilized lipases retained more than 55% of their initial activities after incubation in tert-butanol or chloroform.

This table shows also that the three forms of immobilized lipases do not possess the same stability to organic solvents. Indeed, the immobilized wild type (wt-SAL3i) and the recombinant untagged (r-SAL3i) lipases are more stable than the immobilized recombinant tagged one (His_6-SAL3i) in all tested organic solvents.

Seeing that the immobilized *Staphylococcus aureus* lipase forms are more stable than the free ones, it is then important to use these enzymes to test their esterification capacity.

3.8. Butyl oleate synthesis

As we studied the effect of the His-tag and the recombination process on the hydrolyse sense, it's important to study its effect on the synthesis one. Indeed, the ability of the three immobilized *Staphylococccus aureus* lipases to synthesize the butyl oleate was studied.

The conversion percentages were determined after there was no significant change in product concentration and the results are shown in Fig. 6. We can essentially notice that after 24 h of incubation, the highest conversion percentage was obtained with the wt-SAL3 (71%). In contrast, the two recombinant lipases show comparable and very low conversion percentages (20 and 17%, respectively).

We can conclude that the effect of the recombination process is more deleterious than the presence of the N-terminal tag extension on the esterification capacity of the *Staphylococcus aureus* lipase.

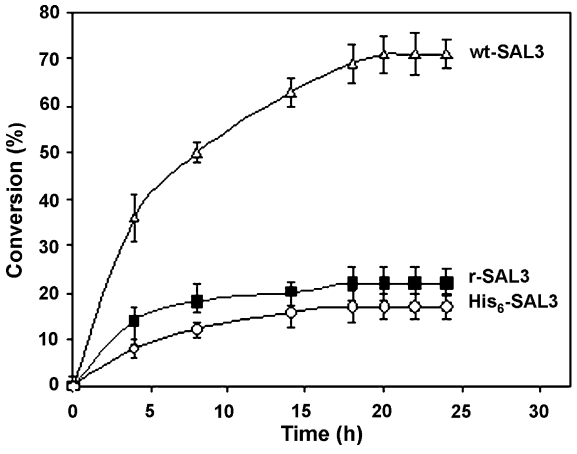

Fig. 6. Kinetic of butyl oleate synthesis by the His_6-SAL3, the r-SAL3 and the wt-SAL3. Reactions were carried out in the presence of hexane using 100 U of each lipase and a concentration of oleic acid and butanol of 0.1 M. Experiments were carried out in triplicates. The S.D. was <5%.

4. Conclusions

The use of affinity tags is preferred as purification can be quick, simple, efficient, and used at large scale. However, the decision regarding the relative positioning and removing of the affinity tags remains difficult and depends on the primary structure sequence and conformity of the protein [30]. Some of the limitations associated with removing tags are: non-specific cleavages generated truncated form of protein, addition of one or two extraaminoacids, partial removal of the tag, and presence of contaminating proteases in the preparation. In addition, the use of affinity tags for protein purification has some perceived limitations which are: misfolding and/or loss of activity or solubility of the protein, inability to use such proteins for X-ray crystallography or other physical characterization studies [31–34]. Many reports show that the His-tag can alter the binding characteristics or structure of recombinant protein when compared to the wild-type protein [35,36]. In addition, polyhistidine affinity tags are small peptides which, in several cases, must be removed by thrombin cleavage from the fusion proteins to allow further analysis of the native part of these proteins.

Furthermore, introducing an affinity tag may have a positive effect in the biochemical properties of the target protein. A recent literature survey on the heat shock protein HSC70 reveals that affinity tags have been observed to improve protein yield [37], prevent proteolysis [38], facilitate protein refolding and increase solubility [39,40].

In the present study, the analysis of the biochemical and kinetic study of three forms of *Staphylococcus aureus* lipases (wild type SAL3, untagged SAL3 and tagged SAL3), reveals that the haxahistidine-tag, when placed at the N-terminus, as well as the recombination process, affect negatively the specific activity, the chain length selectivity and the thermostability. Although the addition of the Histidine-tag to the target protein is a simple and well established approach to facilitate purification, these results emphasize the need to remove the tag before characterization of some recombinant proteins before using them for structural purposes.

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