Identification of amino acids involved in the hydrolytic activity of lipase LipBL from *Marinobacter lipolyticus*

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The lipolytic enzyme family VIII currently includes only seven members but represents a group of lipolytic enzymes with interesting properties. Recently, we identified a gene encoding the family VIII lipase LipBL from the halophilic bacterium *Marinobacter lipolyticus*. This enzyme, like most lipolytic enzymes from family VIII, possesses two possible nucleophilic serines located in an S-X-X-K β-lactamase motif and a G-X-S-X-G lipase motif. The serine in the S-X-X-K motif is a catalytic residue, but the role of serine within the common lipase consensus sequence G-X-S-X-G has not yet been systematically studied. Here, the previously reported time-intensive procedure for purification of recombinant LipBL was replaced by one-step metal-affinity chromatography purification in the presence of ATP. Heterologous co-expression of His6-tagged LipBL with the cytoplasmic molecular chaperones GroEL/GroES was necessary to obtain catalytically active LipBL. Site-directed mutagenesis performed to map the active site of LipBL revealed that mutation of serine and lysine in the β-lactamase motif (S72-M-T-K75) to alanine abolished the enzyme activity of LipBL, in contrast to mutation of the serine in the lipase consensus motif (S321A). Furthermore, mutagenesis was performed to understand the role of the G-X-S-X-G motif and other amino acids that are conserved among family VIII esterases. We describe how mutations in the conserved G-X-S-X-G motif altered the biochemical properties and substrate specificity of LipBL. Molecular modelling results indicate the location of the G-X-S321-X-G motif in a loop close to the catalytic centre of LipBL, presumably representing a substrate-binding site of LipBL.

INTRODUCTION

Microbial esterases and lipases are valuable biocatalysts due to their broad substrate specificity and high chemo-, regio- and stereoselectivity (Chahinian et al., 2005; Houde et al., 2004; Jaeger & Eggert, 2002; Park et al., 2009; Rodriguez et al., 2008; Snellman & Colwell, 2004). Thus, these enzymes are currently used as detergent additives, in the food and paper industries, and as enantioselective biocatalysts for production of fine-grade chemicals (Breuer et al., 2004; Hasan et al., 2006; Jaeger & Eggert, 2002; Jaeger & Holliger, 2010; Jaeger & Reetz, 1998; Snellman et al., 2002; Schmid et al., 2001). However, industrial applications of lipases are often hampered by their low process stability, including low thermostability and loss of activity in organic solvents. One approach to discovering novel enzymes resistant to extremes of salinity, temperature and pH is to study enzymes produced by extremophilic micro-organisms (Niehaus et al., 1999). Although extremophiles are still not commonly exploited, the number of enzymes derived from extremophiles is steadily increasing (Mellado et al., 2005; Tiquia & Mormile, 2010).

Lipolytic enzymes are currently classified into eight families according to conserved motifs and structural and biological properties (Hausmann & Jaeger, 2010). Most of them belong to the α/β-hydrolase fold superfamily of enzymes, and contain a catalytic triad consisting of a nucleophile (Nu), a strictly conserved histidine and an acidic residue. The nucleophilic residue, which attacks the substrate to form an acyl intermediate, is usually located within the conserved motif Sm-X-Nu-X-Sm, where ‘X’ and ‘Sm’...
Lipase from Marinobacter lipolyticus

denote any amino acid and a small amino acid, respectively (Ollis et al., 1992). For most lipases and esterases, the active-site serine residue is located within the G-X-S-X-G motif (Arpigny & Jaeger, 1999; Ollis et al., 1992), forming a sharp γ-turn between a β-strand and an α-helix called a ‘nucleophile elbow’ (Ollis et al., 1992).

In contrast to other microbial esterase families, family VIII esterases are characterized by an active-site serine residue located within the S-X-X-K motif, which is also conserved in class C β-lactamases (Knox et al., 1996) and penicillin-binding proteins (Joris et al., 1988). Only seven family VIII esterase members are currently known, six from different metagenomic libraries (EstM-NI, EstM-N2, EstC, EstCE1, EstA3 and EstU1) and EstB from Burkholderia gladioli (Elend et al., 2006; Jeon et al., 2011; Rashamuse et al., 2009; Wagner et al., 2002; Yu et al., 2011). Site-directed mutagenesis studies performed with EstC, EstCE1, EstA3 and EstB from B. gladioli have revealed complete inactivation of enzyme activity after replacement of serine with alanine in the S-X-X-K motif (Elend et al., 2006; Rashamuse et al., 2009; Wagner et al., 2002; Yu et al., 2011). The crystal structure of EstB from B. gladioli (Wagner et al., 2002) confirmed the function of serine in the S-X-X-K motif as a catalytic nucleophile, and suggested that a conserved lysine in the S-X-X-K motif serves as a proton acceptor for the hydrogen atom of the nucleophile serine during the hydrolytic reaction (Lobkovsky et al., 1993; Oefner et al., 1990).

Curiously, all esterases of family VIII possess an S-X-X-K motif, but only some esterases from this family (EstC, EstA3, EstB) contain a typical G-X-S-X-G lipase motif (Rashamuse et al., 2009; Wagner et al., 2002). Although mutation of this serine to alanine in EstB from B. gladioli leads to a 60 % decrease in enzyme activity, no function for the G-X-S-X-G motif in family VIII esterases has been proposed to date.

In a previous study, we isolated esterase LipBL from the halophilic bacterium Marinobacter lipolyticus (Martin et al., 2003). LipBL is an interesting enzyme due to its high regioselectivity (Pérez et al., 2010, 2011). In this work we aim to understand the roles of evolutionarily conserved catalytic residues of the family VIII esterase LipBL by a combination of site-directed mutagenesis, biochemical methods and homology modelling. We performed site-directed mutagenesis to identify active-site residues and studied the role of the conserved G-X-S-X-G motif in enzyme function for what is believed to be the first time in this family of esterases. We describe the biochemical characterization of recombinant LipBL and mutated variants to elucidate the roles of conserved amino acids in its lipolytic activity and study the biophysical properties of LipBL.

**METHODS**

**Bacterial strains, media and growth conditions.** *Escherichia coli* DH5α (Invitrogen) and *E. coli* BL21(DE3) (Novagen) were used for genetic manipulations and protein overexpression, respectively. These strains were grown in Luria–Bertani (LB) broth (Sambrook et al., 1989) at 30 or 37 °C under aerobic conditions. Ampicillin, kanamycin or chloramphenicol were added, respectively, at 100, 50 or 30 μg ml⁻¹ for selection of plasmids when required.

**DNA manipulation, sequence analyses and structure homologies.** Recombinant DNA manipulations were carried out as described by Sambrook & Russell (2001). Restriction endonucleases (Amersham Biosciences), dephosphorylase and T4 DNA ligase (Promega) were used according to the manufacturers’ recommendations.

The nucleotide and amino acid sequences were analysed using BLAST at the NCBI website (Altschul et al., 1997; Camacho et al., 2009). Sequence alignments were performed with CLUSTAL W (Jeanmougin et al., 1998), Multialign software (version 1.8) and BioEdit software (Hall, 2007).

The 3D structure of LipBL was modelled in Phyre (Protein Homology Analogy Recognition Engine) (Kelley & Sternberg, 2009). The best template was *B. gladioli* esterase (PDB code: 1CI9), with 27 % sequence identity, an E-value of 1 × 10⁻25 and a confidence level of 100 %. PyMOL software was used for structural alignment, analysis and visualization (DeLano, 2002).

**Construction of pET-LipBL expression plasmid.** The *lipBL* gene was PCR-amplified from the *E. coli* plasmid/vector (Pérez et al., 2011) using primers BL75SF/BL7HdIII2 (Table S1 available with the online version of this paper) to introduce *SacI* and *HindIII* sites at the S' and S″ ends of the gene, respectively. The PCR product and the pET28b+ plasmid were hydroxylised with *SacI* and *HindIII* restriction endonucleases, followed by ligation, to yield the pET-LipBL expression vector. With this approach, the lipase gene coding sequence was fused, in-frame, to a coding sequence for a His₆-tag, thereby allowing overexpression of C-terminal His₆-tagged LipBL. Gene expression in pET-LipBL was under the control of a 17 promoter regulated by IPTG-inducible T7 RNA polymerase.

**Site-directed mutagenesis of the *lipBL* gene.** Amino acid substitutions in LipBL were performed by the quick change PCR method using *Pfu* DNA polymerase (Invitrogen), with the pET-LipBL plasmid as template and complementary mutagenic oligonucleotide pairs (Table S1). PCR products were hydroxylised with DpnI restriction endonuclease to remove methylated parental template DNA, then transformed into *E. coli* DH5α, followed by selection of clones with mutations. The presence of desired nucleotide substitutions was confirmed by DNA sequencing.

**Heterologous expression of LipBL and variants.** Overnight cultures of *E. coli* BL21(DE3) harbouring pET-LipBL and pET-LipBL-variant expression vectors were used to inoculate 50 ml LB broth supplemented with kanamycin (30 μg ml⁻¹). Cultures were incubated under aerobic conditions at 37 °C to OD₆₀₀ 0.6, followed by induction with IPTG (1 mM final concentration) and further incubation under the same conditions for 3 h at 30 °C. Harvested cells (7500 g, 10 min, 4 °C) were resuspended in 50 mM Tris/HCl, pH 8.0. Cells were disrupted by two cycles of sonication for 2 min followed by two centrifugation steps, first at 5000 g for 1 min (4 °C) to remove intact cells and cell debris and then at 16 000 g for 10 min (4 °C) to collect inclusion bodies. The supernatants were used as a source of intracellular soluble proteins.

**Co-expression of LipBL and variants with molecular chaperones in *E. coli* BL21(DE3).** LipBL and variants were co-expressed with five different chaperone combinations available in the Takara chaperone kit (Takara Bio). The co-expression system using *E. coli* BL21(DE3) was constructed according to the manufacturer’s instructions. Five expression strains, each containing the pET-LipBL
plasmid in combination with a pG-KJE8, pGro7, pKJE7, pG-T2 or pTrf6 chaperone plasmid, were constructed. Strains containing chaperone plasmids and plasmid pet28b were used as negative controls.

To induce expression of different chaperone combinations, l-arabinose (0.5 mg ml⁻¹) or tetracycline (8 ng ml⁻¹) was added, as required. After this, lipBL was expressed as described above. For the E. coli/pET-LipBL/pGro7 clone, 0.5 mg arabinose ml⁻¹ was initially added to induce expression of the chaperone system and the inoculated medium was then incubated under aerobic conditions at 37 °C to OD₆₀₀ 0.6, followed by induction with IPTG (1 mM final concentration) and incubation under aerobic conditions for 3 h at 30 °C to induce expression of LipBL.

**SDS-PAGE analysis.** SDS-PAGE analyses were performed according to the method of Laemmli (1970) in a Bio-Rad electrophoretic unit using discontinuous polyacrylamide gels (12% separation and 5% to 30%) to the method of Laemmli (1970) in a Bio-Rad electrophoretic unit with activity assays. Protein concentrations were determined after filter units with 10 kDa size-exclusion membranes (Centricon, variants were diluted 50-fold and concentrated using centrifugal purification using Advanced Image Data Analysis (AIDA) 2D software. SDS-PAGE analysis was performed in triplicate.

**Lipase and esterase activity assays.** Hydrolysis of p-nitrophenyl (p-NP) esters. Assays were performed by measuring the increase of absorbance at 410 nm as a result of the release of p-NP upon hydrolysis of substrates (Winkler & Stuckmann, 1979; Lešičić Ailer et al., 2010). The substrates p-NP acetate (C2), p-NP butyrate (C4), p-NP hexanoate (C6), p-NP octanoate (C8), p-NP decanoate (C10), p-NP laurate (C12), p-NP myristate (C14) and p-NP palmitate (C16) were obtained from Sigma-Aldrich. Substrate emulsions contained 1 mM of each p-NP ester, 100 mM potassium phosphate buffer, pH 7.4, 10 mM MgSO₄ and DMSO (5%, v/v). Enzyme reactions were started by adding 5 μl of lipase samples to 200 μl of substrate emulsions in a 96-well microtitre plate, and the plate was incubated at 30 °C. Enzyme activities were calculated using the molar absorption coefficient of p-NP (ε₅₁₅₀ M⁻¹ cm⁻¹). One unit (U) of LipBL activity was defined as the amount of enzyme necessary to hydrolyse one micromole of p-NP ester per minute under the described conditions.

**Hydrolysis of glycerol esters.** The substrate specificity of LipBL and its variants toward triglycerides with different fatty acid chain lengths was determined using a pH-stat device (Methrom). Hydrolysis reactions with each substrate were carried out for 30 min and released fatty acids were neutralized with 0.1 M NaOH at 30 °C to maintain constant pH. Tributyrin (tri-C4), tricaprin (tri-C6), tricaprin (tri-C10), trilaurin (tri-C12), tripalmitin (tri-C16) and triolein (tri-C18:1) were used as substrates (20 mM) in Tris/HCl, pH 7.0, containing 100 mM CaCl₂ and gum arabic (0.5%, w/v).

**β-Lactamase activity assay.** β-Lactamase activity was measured using CENTA (Calbiochem), a chromogenic substrate from thecephalosporin group, which is used for determining the activity of numerous β-lactamases (Bebrone et al., 2001). Substrate solution was prepared by dissolving the sodium salt of CENTA in 100 mM potassium phosphate buffer, pH 7.2, yielding a final CENTA concentration of 150 μM. Enzyme reactions were initiated by combining 10 μl of sample with 150 μl of substrate in a 96-well plate. Absorbance at 405 nm was continuously recorded at 30 °C. An absorption coefficient of ε₅₄₀ M⁻¹ cm⁻¹ for the hydrolytic product released upon hydrolysis of CENTA was used for calculations.

**Biochemical characterization of LipBL and variants.** Thermostability. The stability of LipBL and variants in 50 mM Tris/HCl, pH 8, was determined after incubation at 30, 37, 50 and 60 °C for up to 48 h using p-NP butyrate as substrate.

**pH optimum.** Activities of LipBL and variants were measured at pH 2.0-12.0 using p-NP caprylate as substrate. Control reactions were carried out without addition of enzyme, and their average value was subtracted from each measurement. The maximal activity of each enzyme was considered to be 100%. Sodium acetate buffer (25 mM) was used for the pH range 2.0-6.0, 25 mM sodium phosphate buffer for pH 6.0-8.0 and 25 mM sodium bicarbonate buffer for pH 8.0-12.0.

**Resistance to high salt concentrations.** Activities of LipBL and variants in the presence of NaCl concentrations from 0 to 3 M were measured using p-NP butyrate as substrate. Activities in the absence of NaCl were considered to be 100%. Control reactions were carried out without addition of enzyme and their average value was subtracted from each measurement.

**Resistance to organic solvents.** Activities of LipBL and variants after incubation for 30 min at room temperature with 30% (v/v) DMSO, NN-dimethyl formamide, methanol, acetonitrile, ethanol, diethyl ether, acetone, propan-1-ol, propan-2-ol or 5% (v/v) hexane and toluene in 50 mM Tris/HCl buffer, pH 8.0, were measured. Activities toward p-NP butyrate were measured and expressed as residual activities of LipBL samples in 25 mM sodium phosphate buffer assayed under the same experimental conditions. In all cases, each analysis was performed in triplicate.

**RESULTS**

**Sequence analysis of LipBL for selection of residues for mutagenesis.** The structural lipBL gene encodes a lipase (Fig. S1) showing amino acid sequence homology to both class C β-lactamas and family VIII esterases (Fig. 1). The S²⁷-M-T-K sequence found in LipBL is conserved within class C β-lactamas, penicillin-binding proteins (Joris et al., 1988) and family VIII carboxylesterases (Arpigny & Jaeger, 1999). This sequence resembles the S-X-X-K consensus motif, which includes the active-site serine in these enzymes. Furthermore, the G-L-S³²⁻¹-V-G sequence identified in the LipBL primary structure resembles a classical lipase G-X-S-X-G motif, which harbours catalytic serine in virtually all lipase families (Bornscheuer, 2002) (Figs 1 and S1). In contrast to the S-X-X-K motif conserved in all family VIII
esterases and class C β-lactamases, the G-X-S-X-G motif appears only in LipBL, EstB, EstC and EstBL enzymes classified as family VIII lipases (Fig. 1a).

Based on this primary structure analysis, residues S\textsuperscript{72} and S\textsubscript{321} of LipBL were identified as putative active-site nucleophiles involved in hydrolytic reactions (Kelly & Kuzin, 1995; Kuzin et al., 1995). Because little is known about amino acids other than active-site residues in family VIII esterases, we selected some evolutionarily conserved residues based on alignments of LipBL with homologous β-lactamases (Fig. 1b) and family VIII esterases (Fig. 1a) to target them by site-directed mutagenesis. Residues important for enzyme function are evolutionarily more conserved than non-functional residues (Thornton et al., 1999). Accordingly, five conserved amino acids, namely S\textsuperscript{72}, K\textsuperscript{75}, R\textsuperscript{296}, S\textsubscript{321} and R\textsuperscript{396} (Table 1), were substituted by alanine. Additionally, putative catalytic residues S\textsuperscript{72}, from the S-X-X-K motif, and S\textsubscript{321}, from the G-X-S-X-G lipase-pentapeptide, were substituted by methionine, aspartate and...
activity

Table 1. Mutated residues and their influence on hydrolytic activity

+ , Active variant; −, inactive variant.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Position</th>
<th>Lipolytic activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>S→A</td>
<td>72</td>
<td>−</td>
</tr>
<tr>
<td>S→M</td>
<td>72</td>
<td>−</td>
</tr>
<tr>
<td>S→D</td>
<td>72</td>
<td>−</td>
</tr>
<tr>
<td>S→H</td>
<td>72</td>
<td>−</td>
</tr>
<tr>
<td>K→A</td>
<td>75</td>
<td>−</td>
</tr>
<tr>
<td>R→A</td>
<td>296</td>
<td>+</td>
</tr>
<tr>
<td>H→Q</td>
<td>310</td>
<td>+</td>
</tr>
<tr>
<td>S→A</td>
<td>321</td>
<td>+</td>
</tr>
<tr>
<td>S→M</td>
<td>321</td>
<td>+</td>
</tr>
<tr>
<td>S→D</td>
<td>321</td>
<td>+</td>
</tr>
<tr>
<td>S→H</td>
<td>321</td>
<td>+</td>
</tr>
<tr>
<td>R→A</td>
<td>396</td>
<td>+</td>
</tr>
</tbody>
</table>

Lipolytic activity was measured with p-NPB.

Expression and purification of recombinant LipBL and variants

Recombinant LipBL variants were produced as inactive enzymes in the form of inclusion bodies in *E. coli* BL21(DE3) (Fig. 2a). Only the S72D variant was not successfully expressed, as judged by SDS-PAGE (Fig. 2a) and lipase activity assay (data not shown). Therefore, five different chaperones (pG-KJE8, pGro7, pKJE7, pG-Tf2, pTf16) from the Takara Chaperone kit (Nishihara et al., 1998, 2000) were co-expressed separately, with LipBL and variants in *E. coli* BL21(DE3), for proper folding in vivo. Three hours after simultaneous induction of chaperone and LipBL expression at 30 or 37 °C, LipBL activity was determined using p-NP butyrate as substrate. The highest yields of enzymically active LipBL in the soluble fraction of a cell lysate were obtained after co-expression of LipBL with chaperone plasmid pGro7 at 30 °C. Under these conditions, a 20-fold increase of lipase activity with respect to LipBL expressed without chaperones was observed. After IMAC purification (see Methods) of the soluble fraction of a LipBL-pGro7 co-expression culture of *E. coli* BL21(DE3), LipBL was eluted together with a 57.3 kDa protein in excess over LipBL. The specific activity of this LipBL preparation was 2447.4 U mg⁻¹, measured with p-NP butyrate as substrate. To improve the purification rate, ATP at a final concentration of 2 mM was added to the soluble fraction of LipBL-pGro7 culture, followed by IMAC purification to destabilize the interaction of LipBL with GroEL, thereby avoiding their simultaneous elution (Fig. 2b). This approach resulted in a significant reduction of chaperone protein in the elution fraction, yielding a LipBL preparation with a twofold excess of LipBL over GroEL, measured densitometrically using AIDA software. The specific activity of this LipBL preparation was 2507.7 U mg⁻¹ using p-NP butyrate as substrate. Hence, similar purity yields were obtained with the LipBL variants chosen for biochemical analysis (Fig. 2b).

Identification of catalytic active-site residues of LipBL

LipBL variants with S⁷² of the S-X-X-K-motif replaced by Ala, Met, Asp or His lost their ability to hydrolyse substrates, in contrast to the variants in which S³²¹ of the G-X-S-X-G motif was replaced by Ala, Met, Asp or His. Variants S321A and S321D were more active towards p-NP butyrate than towards LipBL, whereas variants S321M and S321H were less active than LipBL (Table 2). Furthermore, replacement of the second catalytic residue of β-lactamases, K⁷⁵ from the S-X-X-K motif, by Ala resulted in complete inactivation of LipBL. As expected, double mutants S72A/K75A and K75A/S321A and the triple mutant S72A/K75A/S321A also showed complete loss of lipolytic activity.

Furthermore, three mutations of non-catalytic residues, namely R296A, H310Q and R396A, caused partial loss of enzyme activity compared with LipBL (Table 1). According to the 3D model of LipBL, H³¹⁰ and S³²¹ from the G-X-S-X-G motif are located in a loop close to the active site of LipBL (Fig. 3). Two other residues, K⁹⁰⁶ and R⁹⁰⁶, are located on the protein surface far from the active site (Fig. 3). As it is expected that amino acids in the vicinity of the active site might have stronger effects on substrate specificity and enzyme catalysis, we purified and analysed only variants H310Q, S321A, S321M, S321D and S321H. Because the role of the conserved G-X-S-X-G motif in catalysis in family VIII esterases has not been systematically studied, we focused mainly on variants carrying a mutation in the G-X-S⁰³²-X-G motif.

Substrate specificity of LipBL and its variants

LipBL and selected variants (H310Q, S321A, S321M, S321D and S321H) were purified and their specific activities were determined with p-NP esters (Table 2) and triacylglycerol esters (Fig. S2) with fatty acids of various chain lengths. Neither LipBL nor any of its variants showed activity towards esters of long-chain fatty acids with 12, 14 or 16 carbon atoms (data not shown). Generally, the H310Q and S321H variants were 40–100 % less active against all tested p-NP esters compared with LipBL, although the effects on hydrolysis of long-chain p-NP decanoate (C10) were more pronounced (90 and 100 % decreases, respectively) than those on hydrolysis of short-chain p-NP acetate (50 and 38 % decreases, respectively). The S321M mutation resulted in only a slight increase (1–12 %) of activity...
against long-chain (C8 and C10) esters, but it significantly (34–37 %) decreased the activity against short-chain (C2 and C4) esters. The S321D mutation led to an increased activity (97 %) against the short-chain ester p-NP acetate (C2) and a decreased activity (100 %) against long-chain p-NP decanoate (C10).

**Table 2.** Activities of wild-type LipBL and variants using as substrates p-NP esters of fatty acids with 2, 4, 6, 8 and 10 carbon atoms

All values represent means of at least three independent measurements with SDs of less than 5 %.

<table>
<thead>
<tr>
<th>Fatty acid chain length</th>
<th>wt LipBL</th>
<th>H310Q</th>
<th>S321A</th>
<th>S321M</th>
<th>S321H</th>
<th>S321D</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2</td>
<td>952.8</td>
<td>473.3 (50 %)</td>
<td>1454.5 (153 %)</td>
<td>603.0 (63 %)</td>
<td>591.5 (62 %)</td>
<td>1875.7 (197 %)</td>
</tr>
<tr>
<td>C4</td>
<td>2507.7</td>
<td>673.1 (27 %)</td>
<td>2613.8 (104 %)</td>
<td>1644.5 (66 %)</td>
<td>620.3 (25 %)</td>
<td>2732.1 (109 %)</td>
</tr>
<tr>
<td>C6</td>
<td>3016.8</td>
<td>1225.5 (41 %)</td>
<td>2335.9 (77 %)</td>
<td>2819.5 (93 %)</td>
<td>925.6 (31 %)</td>
<td>2186.3 (72 %)</td>
</tr>
<tr>
<td>C8</td>
<td>1747.3</td>
<td>580.6 (33 %)</td>
<td>2009.5 (115 %)</td>
<td>1963.6 (112 %)</td>
<td>626.7 (36 %)</td>
<td>1468.6 (84 %)</td>
</tr>
<tr>
<td>C10</td>
<td>352.2</td>
<td>34.5 (10 %)</td>
<td>652.9 (185 %)</td>
<td>356.4 (101 %)</td>
<td>0.0 (0 %)</td>
<td>0.0 (0 %)</td>
</tr>
</tbody>
</table>

*Values in parentheses represent relative activities expressed as a percentage of wild-type (wt) LipBL activity.
With respect to the size and charge of amino acid side chains, replacing small, hydrophilic serine with large, charged histidine negatively influenced LipBL activity, an effect opposite to replacement with the small, hydrophobic residue alanine, which positively influenced LipBL activity. The S321A variant that showed increased enzyme activity against p-NP esters compared with LipBL was studied using a range of natural triacylglycerol esters. In these assays, significant differences between the activities of wild-type LipBL and S321A were not observed, with the exception of tricaproin (C6), which was hydrolysed by wild-type LipBL with 30% higher preference compared with hydrolysis by S321A (Fig. S2). Comparison of p-NP ester and triacylglycerol ester substrates revealed that both wild-type LipBL and S321A hydrolysed all tested triacylglycerol esters (C4, C6, C10, C12, C16), including unsaturated trioleoylglycerol (C18:1) and olive oil (Fig. S2), while synthetic long-chain p-NP esters (C12, C14, C16) were not hydrolysed by wild-type LipBL. Furthermore, a higher activity of wild-type LipBL was observed for both substrates with C6 fatty acid chains, p-NP hexanoate (23%) and tricaproin (30%).

The sequence similarity of LipBL to a number of class C β-lactamases prompted us to investigate the activity of this enzyme towards β-lactam substrates. However, LipBL was unable to hydrolyse the β-lactam ring of the common chromogenic β-lactamase substrate CENTA (Calbiochem) (Bebrone et al., 2001).

**Biochemical properties of LipBL and variants**

The influence of industrially relevant parameters such as temperature, pH, salt and organic solvents on LipBL enzyme activity was examined by comparing wild-type LipBL with variants H310Q, S321A, S321M, S321D and S321H using p-NP butyrate as substrate.

Thermostability of LipBL. LipBL retained more than 70% of its activity after incubation at 30 °C for 1 h but lost almost 60% of its activity after incubation for 4 h at the same temperature (Table 3). A temperature increase to 37 °C did not significantly change the inactivation profile compared with 30 °C (Table S2), while a temperature increase to 50 °C significantly inactivated LipBL, as it lost 70% activity after 1 h incubation and 90% after 4 h incubation. The activities of enzyme samples that were kept on ice were taken as 100%.

Variant S321A showed a significantly increased thermostability at 30 and 50 °C, as did S321D after incubation at 50 °C for 1 and 4 h (Table 3). Although the precise role of S321 in the thermal stability of LipBL is still unknown, our data suggest that mutation of S321 influences the thermal stability of LipBL.

**pH optimum of LipBL.** The pH optimum of LipBL and its variants was studied in the pH range of 2–12 using p-NP caprylate as substrate because p-NP butyrate exhibits a much lower stability at higher pH values compared with p-NP caprylate (Fig. S3). Neither wild-type LipBL nor any of its variants showed an optimum activity in the pH range of 7–11, and the enzymes were found to be stabilized in this range.

**Table 3. Thermostability of LipBL and variants at 30 and 50 °C with p-NP butyrate as substrate**

<table>
<thead>
<tr>
<th>Variant</th>
<th>Residual activity (%)*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>30 °C</td>
</tr>
<tr>
<td></td>
<td>1 h</td>
</tr>
<tr>
<td>LipBL</td>
<td>71.2</td>
</tr>
<tr>
<td>H310Q</td>
<td>48.1</td>
</tr>
<tr>
<td>S321A†</td>
<td><strong>93.7</strong></td>
</tr>
<tr>
<td>S321M</td>
<td>64.1</td>
</tr>
<tr>
<td>S321H</td>
<td>53.1</td>
</tr>
<tr>
<td>S321D†</td>
<td>53.9</td>
</tr>
</tbody>
</table>

*Residual lipase activity measured after incubation of the enzyme at the given temperature for a certain period is expressed as a percentage of the activity of the protein incubated on ice.
its variants was active at pH 2, 4 or 6. Furthermore, pH 12 completely inactivated LipBL. The optimum pH of wild-type LipBL and variants H310Q, S321A, S321M and S321H was pH 8. The substitution of S321 with aspartate changed the pH optimum of LipBL to pH 10.

Resistance of LipBL to high concentrations of NaCl. LipBL and its variants had the highest activity in the absence of salt (Fig. S4). The presence of 0.5 M NaCl reduced enzyme activity to 60–80 % for wild-type LipBL and H310Q, S321H and S321D. However, under the same conditions, the S321A and S321M variants maintained 82 and 90 % of their activity, respectively (Fig. S4). In general, variants S321A and S321M were more stable than LipBL, retaining approximately 20 % of their activities at 2 M NaCl, compared with only 5 % for LipBL.

Resistance of LipBL to organic solvents. LipBL was activated in the presence of different hydrophobic (high log \( P \)) and hydrophilic (low log \( P \)) solvents (Table 4). Notably, the activity of LipBL increased almost twofold in the presence of propan-2-ol or toluene. Among the solvents tested, only acetonitrile negatively affected LipBL activity, decreasing it to 13 %. The susceptibility of LipBL to acetonitrile was not observed for variants S321A, S321M, S321H or S321D, indicating that these mutations increase the stability of LipBL in 30 % acetonitrile (Table 4). All mutated variants were stable in the organic solvents tested, particularly the S321A and S321M variants, which had significantly increased stability in acetonitrile, dimethyl formamide, methanol and diethyl ether. Interestingly, the activity of the H310Q variant decreased by 44 % in acetonitrile and by 82 % in propan-1-ol. The relative stability of LipBL and its variants in various water/organic solvent mixtures suggests that this enzyme could be used for a variety of organic synthesis reactions.

### DISCUSSION

Family VIII esterases are an unexploited group of lipolytic enzymes, of which only seven members have been experimentally studied (Elend et al., 2006; Jeon et al., 2011; Kim et al., 2010; Petersen et al., 2001; Rashamuse et al., 2009; Yu et al., 2011). Family VIII esterases are evolutionarily unrelated to other esterases, but they share active-site motifs with the \( \beta \)-lactamase family. The family VIII catalytic residues have been studied, but no data exist regarding the function of any other residues for enzyme catalysis. Therefore, we examined the roles of amino acids conserved among family VIII esterases in the activity of the \( M. \) lipolyticus esterase LipBL.

Expression of C-terminal His\(_{6}\)-tagged LipBL under various conditions (30 and 37 °C) in \( E. \) coli BL21(DE3) yielded catalytically inactive LipBL inclusion bodies (data not shown). Therefore, in vivo co-expression of LipBL with the chaperone GroEL/GroES, which assists in folding de novo-synthesized proteins and disaggregating and refolding improperly folded proteins trapped in insoluble aggregates (Ewalt et al., 1997; Hartl & Hayer-Hartl, 2002), yielded catalytically active LipBL in \( E. \) coli BL21(DE3). Co-expression of GroEL has also been successfully applied to enhance the functional expression of other lipases (Narayanan et al., 2011; Shuo-Shuo et al., 2011) in \( E. \) coli. We have previously reported a laborious four-step protocol for chromatographic purification of recombinant LipBL.

### Table 4. Stability of LipBL and variants in the presence of various organic solvents

All values represent means of at least three independent measurements with sds of less than 5 %.

<table>
<thead>
<tr>
<th>Organic solvent</th>
<th>Concentration (% v/v)</th>
<th>Log ( P^* )</th>
<th>LipBL</th>
<th>S321A</th>
<th>S321M</th>
<th>S321H</th>
<th>S321D</th>
<th>H310Q</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>30</td>
<td>–1.300</td>
<td>132.5</td>
<td>136.6</td>
<td>136.7</td>
<td>96.8</td>
<td>121.3</td>
<td>118.5</td>
</tr>
<tr>
<td>N,N-Dimethyl formamide</td>
<td>30</td>
<td>–1.000</td>
<td>118.8</td>
<td>131.2</td>
<td>128.9</td>
<td>120.2</td>
<td>119.0</td>
<td>118.2</td>
</tr>
<tr>
<td>Methanol</td>
<td>30</td>
<td>–0.760</td>
<td>153.4</td>
<td>150.1</td>
<td>174.7</td>
<td>114.2</td>
<td>114.5</td>
<td>129.9</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>30</td>
<td>–0.330</td>
<td>86.9</td>
<td>107.9</td>
<td>110.3</td>
<td>111.8</td>
<td>101.7</td>
<td>55.6</td>
</tr>
<tr>
<td>Ethanol</td>
<td>30</td>
<td>–0.240</td>
<td>148.1</td>
<td>153.5</td>
<td>141.1</td>
<td>125.1</td>
<td>130.2</td>
<td>126.5</td>
</tr>
<tr>
<td>Acetone</td>
<td>30</td>
<td>–0.230</td>
<td>167.9</td>
<td>138.9</td>
<td>132.8</td>
<td>137.2</td>
<td>140.8</td>
<td>126.0</td>
</tr>
<tr>
<td>Propan-1-ol</td>
<td>30</td>
<td>0.280</td>
<td>128.5</td>
<td>129.5</td>
<td>106.5</td>
<td>92.8</td>
<td>112.9</td>
<td>17.9</td>
</tr>
<tr>
<td>Propan-2-ol</td>
<td>30</td>
<td>0.074</td>
<td>187.2</td>
<td>151.0</td>
<td>151.9</td>
<td>160.6</td>
<td>155.3</td>
<td>138.5</td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>30</td>
<td>0.850</td>
<td>149.8</td>
<td>167.2</td>
<td>176.3</td>
<td>161.8</td>
<td>135.5</td>
<td>141.1</td>
</tr>
<tr>
<td>Hexane</td>
<td>5</td>
<td>3.500</td>
<td>141.2</td>
<td>148.8</td>
<td>115.4</td>
<td>117.4</td>
<td>121.8</td>
<td>120.3</td>
</tr>
<tr>
<td>Toluene</td>
<td>5</td>
<td>2.500</td>
<td>181.8</td>
<td>131.8</td>
<td>138.8</td>
<td>118.6</td>
<td>109.2</td>
<td>111.2</td>
</tr>
</tbody>
</table>

\( ^* \) Log \( P \) values according to Laane et al. (1987).

\( ^\dagger \) Residual lipase activity measured after incubation of the enzyme with organic solvent for 30 min is expressed as a percentage of LipBL activity in buffer without organic solvent.

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expressed in E. coli DH5α (Pérez et al., 2011). Here, we describe a simplified and improved one-step metal-affinity chromatography purification of LipBL tagged with a C-terminal poly-His tail and co-expressed with GroEL in E. coli BL21(DE3). Purification of LipBL by metal-affinity chromatography yielded 78% LipBL, in contrast to the 60% purification yield from the previously described four-step purification. Although we have observed a minor influence of the poly-His tail on the pH optimum of LipBL (Pérez et al., 2011), it has no effect on the substrate specificity of LipBL or on its resistance to organic solvents and high salt concentrations.

We also demonstrated that LipBL, although similar in sequence to class C β-lactamases, does not exhibit β-lactamase activity. In esterase family VIII, only EstC (Rashamuse et al., 2009), EstM-NI and EstM-N2 (Yu et al., 2011) have been proven to have β-lactamase activity. It has been speculated that the lack of β-lactamase activity in family VIII esterases could be due to steric constraints (Wagner et al., 2002).

After comparative analysis of the LipBL primary structure, we proposed two serine residues (S72 and S321) as catalytic amino acids and analysed the roles of these residues by mutating them to alanine, methionine, aspartate or histidine. Serine-to-alanine substitutions often lead to enzyme inactivation (Holm et al., 1994; Rashamuse et al., 2009) but minimize disturbances in protein structure (Bordo & Argos, 1991). All substitutions of S72 completely abolished enzyme activity, whereas substitutions of S321 had only a modest influence on LipBL activity. These data clearly point to S72 in the β-lactamase S-X-X-K-motif as a catalytic residue, rather than S321 from the G-X-S-X-G motif. These results were further confirmed by our complete inactivation of LipBL by substituting alanine for a second catalytic residue, K75, which deprotonates the nucleophilic serine during catalysis (Lobkovsky et al., 1993; Oefner et al., 1990). A similar result has been reported for two family VIII esterases of metagenomic origin, EstC and EstU1, which lose activity after substitution with alanine for serine or lysine from the S-X-X-K motif (Jeon et al., 2011; Rashamuse et al., 2009).

The variants S321D and S321A were threefold more stable at 50°C after 4 h incubation compared with LipBL. Prolonged incubation (24 h, 50°C) decreased the activity of variant S321A to the level of wild-type LipBL, but variant S321D retained 33% of its activity, which was sixfold higher than that of wild-type LipBL. LipBL variant S321D is thus a relatively robust enzyme suitable for further optimization as an industrial biocatalyst for applications at temperatures as high as 50°C. Similar to LipBL, single point mutations in lipases of Staphylococcus simulans (Sayari et al., 2007), Geobacillus sp. RD-2 (Wu et al., 2010) and Staphylococcus xylosus (Mosbah et al., 2007) affect their substrate specificity and thermostability.

In addition, we studied the influence of pH, salts and organic solvents on the stability and activity of LipBL variants. Studies of the enzyme activity of wild-type LipBL and its variants in the presence of NaCl revealed LipBL as an enzyme with low salt tolerance. Furthermore, LipBL has a pH optimum of 8, and the S321D mutation shifted its pH optimum to 10. Although the H310Q, S321M, S321A and S321H mutations had only weak effects on salt tolerance and optimum pH, we have demonstrated that these mutations affect resistance to organic solvents. Extremozymes from micro-organisms isolated from saline environments are resistant to organic solvents due to their natural adaptations to environments with low water activity (Zaccar, 2004). Solvents with high log P values cause less enzyme denaturation than solvents with log P values lower than 2, which distort water–biocatalyst interactions and strip essential water from enzymes (Laane et al., 1987). In our study, we showed that LipBL is quite stable in the presence of various industrially relevant organic solvents (Table 4) and is actually activated by most organic solvents. The strongest activation was observed with propan-2-ol and toluene, at 87 and 81%, respectively. Among mutated variants, S321A and S321M were particularly stable in the organic solvents tested. These data provide more information about the effect of S321 mutations on the flexibility of the enzyme, as proposed above. Due to the biotechnological significance of the issue, several studies have been reported on hydrolases that are highly stable in solutions containing water-miscible organic solvents (Ogino & Ishikawa, 2001).

Our findings clearly indicate the importance of residue S321 for the thermal stability and substrate specificity of LipBL. The S321A and S321D variants showed the highest specificity towards p-NPB, in contrast to wild-type LipBL, which showed the highest specificity towards p-NP hexanoate (Table 2). This suggests that the S321 residue is located near the active centre of LipBL. Analysis of mutated residues using the 3D model of LipBL (Fig. 3) suggested that S321 and H310 are located in a surface-exposed loop (His310–Ser321) near the active-site cavity. Therefore, it is reasonable to assume that mutations of S321 and H310 might lead to local conformational changes of the proposed substrate-binding loop, thereby changing its rigidity and affecting the substrate specificity and/or thermal stability of LipBL. E. coli esterase (Huang et al., 2001) and Humicola lanuginosa lipase (Peters et al., 1998) are examples of enzymes that exert conformational changes of flexible loops around the active site upon interactions with substrate. Furthermore, mutagenesis studies of EstB, a family VIII esterase from B. gladioli with a known crystal structure, have revealed mutations that enhance enzyme stability (Valinger et al., 2007). Curiously, three mutations are in the flexible loop (Arg308–Pro320) located in the vicinity of the active site, which is structurally equivalent to the proposed substrate-binding loop of LipBL, which contains His310 and Ser321 residues. Those authors propose a stabilization of this loop by additional interactions of mutated residues as an explanation of the increased thermal stability of EstB variants (Valinger et al., 2007). To obtain greater insight into the roles of mutated amino acids in the catalytic mechanism of LipBL, an X-ray structure will...
be necessary, which has prompted us to initiate the crystallization of LipBL.

In summary, we have shown that LipBL contains a single active site (S\(^{22}\)-M-T-K\(^{75}\)) responsible for catalytic activity. In addition we have, we believe for the first time, demonstrated the role of S\(^{321}\) in the conserved G-X-S-X-G sequence motif in the functionality of a lipase family VIII enzyme. We propose that loop H\(^{310}\)-S\(^{321}\) is located in the vicinity of the active site of LipBL and interacts with substrates during hydrolysis. Moreover, we have identified mutations that affect the stability of LipBL in the presence of organic solvents (S321A and S321M), at high temperatures (S321D and S321A) and at extreme pH (S321D). We conclude that our rational engineering strategy allowed us to identify several LipBL variants with altered catalytic properties that could be useful when different temperatures, pHs and substrates are needed.

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