Selection of novel lipases and esterases for enantioselective biocatalysis
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Chapter 6

BINDING OF PHAGE DISPLAYED *Bacillus subtilis* LIPASE A TO A PHOSPHONATE SUICIDE INHIBITOR

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Phage display of Bacillus subtilis lipase A

Binding of phage displayed Bacillus subtilis lipase A to a phosphonate suicide inhibitor

Phage display can be used as a protein engineering tool to select proteins with desirable binding properties from a library of randomly constructed mutants. Here, we describe the development of this method for the directed evolution of Bacillus subtilis lipase A, an enzyme that has marked properties for the preparation of pharmaceutically relevant chiral compounds. The lipase gene was cloned upstream of the phage g3p encoding sequence and downstream of a modified g3p signal sequence. Consequently, the enzyme was displayed at the surface of bacteriophage fd as a fusion to its minor coat protein g3p. The phage-bound lipase was correctly folded and fully enzymatically active as determined from the hydrolysis of p-nitrophenylcaprylate with Km values of 0.38 mM and 0.33 mM for the phage displayed and soluble lipase, respectively. Both soluble lipase and lipase expressed on bacteriophages reacted covalently with a phosphonate suicide inhibitor. The phage does not hamper lipase binding, since both soluble and phage-bound lipase have a similar half-life of inactivation of approximately five minutes. Therefore, we conclude that the Bacillus lipase can be functionally expressed on bacteriophages as a fusion to the phage coat protein g3p. The specific interaction with the suicide inhibitor offers a fast and reproducible method for the future selection of mutant enzymes with an enantioselectivity towards new substrates.

Introduction

The introduction of protein engineering techniques has allowed the creation of novel enzymes and proteins. Besides the increased knowledge of structure-function relationships of proteins, this has rendered several practical applications (Marrs et al., 1999). For example, lipases have become of commercial importance in organic synthesis and as constituents of washing detergents (Jaeger et al., 1994; Reetz & Jaeger, 1998; Schmidt-Dannert, 1999; Soberón-Chávez & Palmeros, 1994). Moreover, lipases have been engineered in particular for the environmentally friendly and cost-effective preparation of pharmaceutically relevant chiral compounds (Koeller & Wong, 2001; Liebeton et al., 2000; Schmid et al., 2001). However, the success of the most familiar approach towards protein engineering, a combination of random mutagenesis and screening, is frequently restricted by the size of the library to be screened and the rather limited throughput of screening assays. Phage display is a well established method for the selection of small peptides and proteins on filamentous phages, by inserting the gene of interest into the phage gene g3p sequence (Smith, 1985). The power of this technique lies in the ability to test many variants...
with subtle differences in a short period of time, as shown firstly in the field of antibody fragments (for a review see Wilson & Finlay, 1998). Recently, the phage display technology was employed to select enzyme variants with enhanced biophysical properties or catalysts with improved or novel activities on suicide inhibitors (Avalle et al., 1997; Danielsen et al., 2001; Soumilion et al., 1994; Vanwetswinkel et al., 1995; 1996), transition state analogues (Widersten et al., 2000) or substrates anchored to the phage (Atwell & Wells, 1999; Demartis et al., 1999; Jestin et al., 1999).

Bacterial lipases (E.C. 3.1.1.3.) with potential industrial application have thus far been found in *Pseudomonas*, *Thermomyces*, and *Staphylococcus* species (Danielsen et al., 2001; Reetz & Jaeger, 1998; Schmidt-Dannert, 1999; Soberón-Chávez & Palmeros, 1994). However, functional expression of these lipases on bacteriophages and subsequent selection of variants with improved characteristics is rather complicated (Danielsen et al., 2001). Our previous attempts to display the lipases of *Pseudomonas aeruginosa* and *Pseudomonas alcaligenes* were unsuccessful (data not published). Most likely, the failure to display active lipases on bacteriophages is related to their complex structure, the presence of a lid, and the requirement of specific chaperones and modifications for their activation (Frenken et al., 1993; Hobsin et al., 1993; Jorgensen et al., 1991; Rosenstein & Götz, 2000). The *in vitro* enzymatic activity of those lipases can be measured by the hydrolysis of water insoluble long-chain acylglycerols (Jaeger et al., 1994). Since this type of compound is water insoluble, the conversion to glycerol and organic acids takes place at the water-lipid interface. This process, known as interfacial activation, is likely to be enhanced by the presence of a lid-like polypeptide, which covers the active site of the lipase. After diffusion of the lipase into the interface, the active site cleft of the enzyme is exposed due to a conformational change of the lid (Milled et al., 2001). It was speculated previously that *Bacillus* lipase might lack this characteristic lid, since this enzyme is not activated in the presence of emulsified substrates (Eggert et al., 2000; Jaeger et al., 1994). Recently, this has been confirmed by the elucidation of the crystal structure (Van Pouderoyen et al., 2001). As a consequence, the catalytic activity of this enzyme can be described by Michaelis-Menten kinetics. In this respect, the lipase A (LipA) of *Bacillus subtilis* 168 is of particular interest as it shows more advantageous characteristics: its small size (181 amino acids, 19 kDa) and, unlike other lipases, it can be expressed and engineered in *Escherichia coli* and *B. subtilis*, since it does not require specific chaperones.

The objective of the present study was to demonstrate that the advantageous characteristics of LipA eventuated in a successful display on bacteriophage fd. In addition, we assessed a selection method for lipase expressing phages on a phosphonate suicide inhibitor. This is highly interesting for the future selection of lipase mutants with an enantioselectivity towards new substrates.
Material and methods

Plasmids, bacterial strains and media

_E. coli_ TG-1 (supE, K12 Δ(lac-pro), _thi_, _bzdD5/F’_, _traD36_, _proAB_, _laqI_, _lacZΔ-M15_), pCANTAB 5E were purchased from Pharmacia (Amersham Pharmacia Biotech, Uppsala, Sweden). LB medium contained: Bactotrypton (1% w/v), Bacto yeast extract (0.5% w/v) and sodium chloride (0.5% w/v); 2xTY medium contained: Bactotrypton (1.6% w/v), Bacto yeast extract (1% w/v) and sodium chloride (0.5% w/v). Antibiotic agents (Duchefa Biochemie, Haarlem, The Netherlands) were used in the following concentrations: ampicillin 100 μg.ml⁻¹, kanamycin 50 μg.ml⁻¹.

Chemicals

Genencor International (Leiden, The Netherlands) provided a fermentor broth of the strain _B. subtilis_ 1051, producing LipA (Dartois et al., 1992). Collagenase A was purchased from Roche Diagnostics (Indianapolis, IN, USA) and _p_-nitrophenylcaprylate from Sigma (St. Louis, MO, USA).

Oligonucleotides

To construct the plasmids shown in figure 1, the following primers were used (Department of Organic Chemistry, Leiden University, The Netherlands): LAF2: 5’-CTGTTTGCGTTGGCCCAGCCGGCCATGGCCGCTGAACACAATCC-3’ (_SfiI_), LAR1: 5’-TCAAGGTTTTGTTGCGGCCGCCTTCGTATTCTGGCC-3’ (_NotI_). Newly created restriction sites are indicated in bold italics.

DNA techniques

Recombinant DNA techniques were performed as described by Sambrook et al. (1989). Plasmid DNA was prepared as described by Birnboim & Doly (1979). DNA purification was performed using the Qiaquick Gel Extraction kit (Qiagen, Hilden, Germany).

Construction of the plasmids

The _lipA_ sequence was amplified from the chromosomal DNA of _B. subtilis_ 168 using the LAF2 and LAR1 primers. Note that the signal sequence of the _lipA_ will not be amplified. PCR was performed using _Pfu_ DNA polymerase (Stratagene, La Jolla, CA, USA). To obtain high yields of PCR product, a PCR protocol with low annealing and extension temperatures was used: 4 min at 94°C, followed by 10 cycles of 30 s at 94°C, 1 min 37°C and 10 min at 55°C, followed by 30 cycles in which the extension time was 8 min at first, but increased by 15 s in each cycle (first cycle: 30 s 94°C, 1 min 55°C and then 8 min 72°C). At the end, DNA production was finished for 10 min at 72°C. The amplified gene fragment was digested with _SfiI_ (Pharmacia) and _NotI_ (Pharmacia), and cloned in _E. coli_
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TG-1 into the SfiI and NodI sites of the phagemid pCANTAB 5E (plasmid pCANTABLip-E). Plasmid pCANTABLip-CH was constructed by inserting the SfiI and Eco52I gene-fragment of pCANTABLip-E into a modified pCANTAB vector (Verhaert et al., 1999). After transforming E. coli TG-1, strains Ec(pCANTABLip-E) and Ec(pCANTABLip-CH) were obtained. Prior to phage display, the constructs were sequenced to verify the correct base pair order.

**Phage rescue**

10^{10} helperphages were added to exponential phase growing Ec(pCANTABLip-E) and Ec(pCANTABLip-CH) cells (i.e. phage-to-bacterium ratio 30:1), followed by 16 h of growth at 28°C in a glucose depleted 2xTY medium containing ampicillin and kanamycin. Phages were precipitated by the addition of 4% w/v polyethyleneglycol (PEG4000) in 0.6 M NaCl. After centrifugation, the phages were resuspended in 2 ml 10 mM Tris HCl buffer (pH 7.4) containing 1 mM EDTA, and filtered through a 0.45 µm filter (Millipore, Bedford, MA, USA). The number of phage particles in the suspension was determined by mixing 150 µl of a diluted phage suspension (10^2 – 10^8-fold) with 150 µl exponential-phase E. coli TG-1 cells. After plating of 5 µl of the phage suspension and overnight growth, the number of infective phages was counted. The protein content was also determined by performing a Bradford assay using bovine serum albumin (BSA) as a standard (Pierce, Rockford, Illinios, USA).

**Electrophoresis**

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 12% separating and a 4% stacking gel (Laemmli, 1970). Molecular mass markers were purchased from Bio-Rad. After electrophoresis, proteins were blotted to nitrocellulose and immunostained with a rabbit antiserum against LipA or with mouse monoclonal antibodies against g3p (PSKAN3; MoBiTec, Göttingen, Germany). Detection of the antibody was performed with alkaline phosphatase-conjugated goat antibodies against rabbits (LipA antiserum) or mice (g3p antibody).

**Enzyme kinetics**

LipA was purified as described by Lesuisse et al. (1993). Enzymatic activity was determined spectrophotometrically by the p-nitrophenylcaprylate assay (Lesuisse et al., 1993). A 10 mM solution p-nitrophenylcaprylate in methanol was prepared. 0.5 mM p-nitrophenylcaprylate was added to 900 µl assay buffer, containing 50 mM phosphate buffer (pH 8), 0.36% Triton X100 (v/v) and 0.1% gum arabic. 50 µl purified or phage-bound LipA (1 µg LipA or 1 × 10^{11} phage particles) were added and the absorbance was measured at 410 nm. Concentrations were calculated using a molar extinction coefficient of 15.000 M^{-1}.cm^{-1}. One unit (U) is defined as the amount of enzyme that hydrolyses 1 µmol p-nitrophenylcaprylate per minute. Corrections were made for spontaneous hydrolysis of the
substrate. The Michaelis-Menten constant ($K_m$), specific activities and turnover numbers of the soluble and phage-bound LipA were determined with substrate concentrations between 0.2 mM and 0.5 mM. All data were expressed as mean ± SEM (n=4). The statistical significance of differences was tested at a significance level of p<0.05 using a two-tailed Student's $t$-test.

Determination of the enantioselectivity towards 1,2-O-isopropylidene-$sn$-glycerol (IPG) esters was performed as described previously (Dröge et al., 2001). All data were the results of three experiments. Enantiomeric ratios, $E$, were calculated and were defined as the ability of the enzyme to distinguish between enantiomers (Chen et al., 1982).

**Binding to the suicide inhibitor**

The inhibitor was synthesised according to Reetz et al. (2002c). The inactivation of LipA by the phosphonate inhibitor was determined by incubating the enzyme and the phosphonate inhibitor in assay buffer (50 mM phosphate buffer, pH 8, 0.36% Triton X100 (v/v) and 0.1% gum arabic) at room temperature. Triton X100 was used to reduce the aspecific interaction of the LipA expressing phages with the matrix via hydrophobic interactions. 10 mg of the inhibitor (loading 10 mmol inhibitor per g material) was washed three times and pre-equilibrated for 10 minutes using 500 µl assay buffer. Note that the inhibitor does not dissolve in this solution. Therefore the reaction mixture was vertically rotated during incubation. Subsequently, purified LipA (1 µg) or LipA expressing phages (1 × 10$^{10}$ phages) were added to the inhibitor. As controls, LipA and phage-bound LipA were also incubated in assay buffer without the inhibitor for the same time period. During incubation of the lipase, aliquots of 10 µl were collected to determine the residual LipA activity using the $p$-nitrophenylcaprylate assay, as described above. All results are expressed as mean ± SEM. The statistical significance of differences was tested at a significance level of p<0.05 using a two-tailed Student's $t$-test.

To assess the amount of phages that bound to the inhibitor, the inhibitor was washed and pre-equilibrated as described above. Subsequently, M13KO7 phages, LipA expressing phages or a mixture of LipA expressing and M13KO7 phages were added and allowed to react for 20 minutes. Subsequently, the supernatant was removed and the inhibitor was washed 8 times for 10 minutes to remove all the non-binding and aspecifically bound phages. Afterwards, 0.1% w/v collagenase A or exponential phase growing *E. coli* TG-1 cells were added to the reaction mixture to elute the bound phages or the phagemids, respectively (Wind et al., 1997). The total number of phages in each fraction was determined by phage counting as described above.
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Results

Construction of the lipase phagemids

The LipA encoding gene (Genbank accession number M74010) was cloned in the phagemid pCANTAB 5E, downstream of a modified g3p signal sequence and upstream of an E-tag, an amber stop codon, and the sequence encoding residues 3-406 of the g3p coat protein (figure 1). The original g3p maturation site, SHS, in this phagemid was modified to AAQPAMA in order to better resemble the consensus (Verhaert et al., 1999). In a second construct, the E-tag was replaced by a sequence encoding a collagenase site and six consecutive histidine residues (pCANTABLip-CH, figure 1) (Verhaert et al., 1999). The histidine residues can be used for the purification of wild-type and mutant enzymes. The collagenase cleavage site was used for the elution of the phage with collagenase A after covalent interaction of the phage-bound LipA with the phosphonate inhibitor.

Phage display of Bacillus lipase

Ec(pCANTABLip-E) and Ec(pCANTABLip-CH) were infected with M13KO7 helperphages to produce phage particles containing the phagemid genome and a mixture of wild-type and fusion g3p. The LipA fusion to g3p results from a partial suppression of the TAG stop codon in the host cells E. coli TG-1. SDS-PAGE under reducing conditions and Western blot analysis with a rabbit antiserum against LipA and with mouse monoclonal antibodies against g3p were performed to detect soluble LipA, phage-bound LipA and the g3p protein (figure 2).

**Figure 1:** Schematic representation of the molecular structure of the vector used for the cloning of the genes. The lipA gene was PCR amplified. After SfiI and NdeI digestion the gene was cloned in E. coli into the SfiI and NdeI sites of the phagemid pCANTAB 5E. LipA was respectively fused to an E-tag or a Prot-His6-tag which encoded a collagenase restriction site and a hexahistidine tag.
The soluble enzyme showed a protein band of approximately 19 kDa, corresponding to the molecular weight of the enzyme, and a specific protein band at approximately 40 kDa. The g3p coat protein was visible at an apparent molecular weight of 65 kDa, as expected, (Tesar et al., 1995) in the lanes containing the M13KO7 phages and the LipA phages. The fusion protein was detectable at an apparent molecular weight of approximately 85 kDa, corresponding to the weight of LipA plus a g3p protein. Despite the fact that an amber stop codon was present in the phagemid, western blot analysis of the phage solutions using the rabbit antiserum against LipA showed that LipA is present exclusively in the phage-bound form and not in the soluble form.

**Enzymatic activity of soluble and phage-bound Bacillus lipase**

To investigate whether the kinetic properties of the phage-bound LipA were unaltered, the $K_m$, the specific activities, and the turnover were measured of both soluble and phage-bound LipA. The steady state hydrolysis of $p$-nitrophenylcaprylate showed that the $K_m$ of the enzyme remained unchanged (table 1), while the specific activity of the phage-bound LipA was reduced. Since the observed difference is likely to result from the different molecular weights of the soluble LipA and a phage particle ($3.15 \times 10^{-17}$ and $2.20 \times 10^{-14}$ mg, respectively), we calculated the number of $p$-nitrophenylcaprylate molecules hydrolysed by one molecule of soluble LipA or one single phage particle. The turnover of the soluble LipA was 18 molecules per lipase molecule per second. The catalytic activity of the phage solution was 84 molecules per phage per second, being significantly different (p<0.05).

**Figure 2:** Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) patterns of phagemids displaying LipA. Left panel: immunostaining with a rabbit antiserum against LipA; right panel: immunostaining with mouse monoclonal antibodies against g3p. Lane 1 & 4: $8 \times 10^{10}$ colony forming M13KO7 phages; lane 2 & 5: $7 \times 10^{10}$ colony forming phages containing the LipA sequence (pCANTABLip-E); lane 3: approximately 90 ng purified LipA.
TABLE 1: Enzyme kinetics of LipA from B. subtilis 168.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity (U.mg⁻¹)</th>
<th>Kₘ (mM)</th>
<th>Turnover (molecules.s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble LipA (n=4)</td>
<td>57.5 ± 1.0</td>
<td>0.33 ± 0.09</td>
<td>18 ± 6</td>
</tr>
<tr>
<td>Phage-bound LipA (n=5)</td>
<td>0.38 ± 0.06</td>
<td>0.38 ± 0.06</td>
<td>84 ± 17</td>
</tr>
</tbody>
</table>

Binding to the suicide inhibitor

In order to develop a selection system for phage-bound LipA mutants, a suitable transition state analogue was synthesised (figure 3). This immobilised inhibitor consisted of a substrate analogue, S-(+)-1,2-O-isopropylidene-sn-glycerol ((+)-IPG); a leaving group, p-nitrophenol; and a glass-bead matrix connected to the phosphonate moiety through a linker. The C₁₁ alkyl chain linkage between the phosphonate and the glass-bead matrix was chosen to avoid any possible steric hindrance between the matrix of the inhibitor and the hydrophobic cleft of LipA (Van Pouderoyen et al., 2001). Note that the inhibitor is enantiopure at the chiral atom of the IPG molecule (Sₜ inhibitor) and racemic at the phosphorus atom (mixture of Rp and Sp inhibitor). We determined the residual catalytic activity of LipA during incubation with this phosphonate suicide inhibitor.

Figure 4 shows that the Sₜ inhibitor inactivated 50% of the purified LipA after 5.2 minutes. LipA bound to phages was inactivated by the same Sₜ phosphonate inhibitor with a t₁/₂ of inactivation of 5.0 minutes, being not significantly different from soluble LipA (p>0.05). The controls were incubated in assay buffer without an inhibitor and showed no inactivation.

![Siran](image)

**FIGURE 3**: Structure of the lipase suicide inhibitor. The inhibitor consisted of a phosphonate moiety (a mixture of Sp and Rp), enantiopure (+)-1,2-O-isopropylidene-sn-glycerol, and p-nitrophenol as leaving group. The phosphonate is connected through a linker to a glass-bead (SIRAN) matrix. Note that the absolute configuration of IPG changes upon attachment to the phosphorus atom.
FIGURE 4: Residual activity of LipA during incubation with the phosphonate suicide Sc inhibitor. During incubation samples were taken and the residual lipase activity was determined by the p-nitrophenylcaprylate assay. (●) soluble LipA; (■) phage-bound LipA. All results are expressed as mean ± SEM (n=3).

Subsequently, binding experiments were performed to check whether the phages expressing LipA interacted specifically with the Sc phosphonate inhibitor (table 2). Three different phage samples were incubated with the Sc phosphonate inhibitor: a M13KO7 helperphage solution (carrying the helperphage genome with a kanamycin resistance marker); the LipA expressing phage solution (containing phages with an ampicillin resistant marker and the lipA gene, and phages with a kanamycin resistance marker); and a mixture of helperphages and LipA expressing phages.

Free M13KO7 helperphages did not bind aspecifically to the Sc inhibitor, since infective helperphages could hardly be demonstrated after the addition of exponential phase growing E. coli to the Sc inhibitor. In contrast, phages carrying the phagemid genome and a LipA fusion protein, did bind to the Sc inhibitor, because those phages could be demonstrated after collagenase A digestion or after addition of exponential phase growing E. coli TG-1.

The same was true for phages, carrying the helperphage genome with a kanamycin resistance marker and possibly a LipA fusion, present in the solution with the LipA expressing phages. Typically, the ratio of ampicillin and kanamycin resistance was conserved in all collected fractions of this sample. In contrast, this ratio changed in the mixture containing M13KO7 phages and LipA expressing phages. Phages carrying a kanamycin resistance marker were abundantly present in the wash fraction, resulting in a five-fold decrease of the ratio, whereas an almost thirty-fold increase of the ratio was observed in the eluted fractions (using either collagenase or E. coli TG-1. Therefore, this sample can be enriched for LipA expressing phages.
**Table 2:** Binding experiment. The inhibitor was washed and pre-equilibrated as described in material and methods. Phages were added (t=0) and, after 20 minutes incubation, the non-binding phages were collected. Subsequently, the inhibitor was washed 8 times and collagenase A or exponential phase growing *E. coli* TG-1 were added to the reaction mixture. The number of phages in each fraction was determined by phage counting as described in the material and methods. Note that the detection level of this method is $4 \times 10^2$ phages ml$^{-1}$. Amp: ampicillin resistance; kana: kanamycin resistance; n.d.: not determined.

<table>
<thead>
<tr>
<th>Resistance</th>
<th>Amp</th>
<th>Kana</th>
<th>Amp</th>
<th>Kana</th>
<th>Amp</th>
<th>Kana</th>
</tr>
</thead>
<tbody>
<tr>
<td>t=0</td>
<td>Below detection$^{*1}$</td>
<td>$7.0 \times 10^7$</td>
<td>$1.2 \times 10^{10}$</td>
<td>$2.0 \times 10^7$</td>
<td>$2.2 \times 10^9$</td>
<td>$8.0 \times 10^7$</td>
</tr>
<tr>
<td>Non-binders</td>
<td>Below detection $^{*1}$</td>
<td>$6.4 \times 10^7$</td>
<td>$1.1 \times 10^{10}$</td>
<td>$1.6 \times 10^7$</td>
<td>$2.0 \times 10^9$</td>
<td>$7.7 \times 10^7$</td>
</tr>
<tr>
<td>Wash</td>
<td>Below detection $^{*1}$</td>
<td>$5.8 \times 10^6$</td>
<td>$1.2 \times 10^8$</td>
<td>$2.0 \times 10^6$</td>
<td>$6.4 \times 10^7$</td>
<td>$1.1 \times 10^7$</td>
</tr>
<tr>
<td>Collagenase</td>
<td>Below detection $^{*1}$</td>
<td>n.d.</td>
<td>$1.8 \times 10^7$</td>
<td>$2.0 \times 10^4$</td>
<td>$8.0 \times 10^6$</td>
<td>$1.0 \times 10^4$</td>
</tr>
<tr>
<td>TG-1</td>
<td>Below detection $^{*1}$</td>
<td>Below detection $^{*1}$</td>
<td>$1.6 \times 10^6$</td>
<td>$8.0 \times 10^3$</td>
<td>$8.1 \times 10^6$</td>
<td>$1.0 \times 10^4$</td>
</tr>
</tbody>
</table>

$^{*1}$ below detection: less than $4 \times 10^2$ phages

**Discussion**

Here, we describe a lipase that can be functionally displayed at the surface of phage fd when fused to the phage fd minor coat protein g3p. In general, it is anticipated that the complex process of folding and secreting lipases complicates their expression in a heterologous host. In addition, the interfacially activated catalysis by lipases can be divided into several reaction steps complicating the design of a selection protocol. Recently, this was demonstrated by Danielsen et al. (2001). They showed that the lipase of *T. lanuginosa* could be displayed in active form on phages as a fusion to the phage coat protein g3p, but selection of improved lipase variants failed (Danielsen et al., 2001). In this respect, we hypothesised the LipA of *B. subtilis* to be an important exception. The kinetic data from the present study show that the $K_m$ of the phage-bound LipA towards the substrate $p$-nitrophenylcaprylate remains unchanged, suggesting that the protein is correctly folded and fully enzymatically active, which is a prerequisite for the proper selection of improved mutants. The enhanced turnover number of LipA on the bacteriophage may suggest that more than one LipA molecule is expressed on the surface of each phage. However, estimation of the amount fusion protein by both SDS-PAGE and Western blot analysis...
Phage display of Bacillus subtilis lipase A does not support the presence of multi-displayed lipase. An alternative explanation might be the activation or stabilisation of LipA on the bacteriophage.

Next, we designed a suicide inhibitor for the selection of improved LipA variants on the basis of the reaction mechanism of ester hydrolysis by lipases (Jaeger et al., 1994). Phosphonates can be used as a ligand, since they mimic the first transition state in ester hydrolysis (Deussen et al., 2000a; 2000b; Mannesse et al., 1995; Stadler et al., 1996). This is due to the charge distribution and the geometric configuration of the phosphorus atom, such as the negatively charged oxyanion hole intermediate and sp3 hybridisation of the central atom. Mechanistically, serine hydrolases are inhibited preferably by $Sp$ phosphonates via an in-line displacement reaction, in which the nucleophilic displacement occurs opposite of the leaving group, resulting in an inversion of the configuration (Mannesse et al., 1995).

To determine whether the synthesised phosphonate inhibitor was suitable for the enrichment of phage displayed LipA, we chose to study a substrate containing the chiral intermediate IPG as a starting compound in the synthesis of β-adrenoceptor antagonists. Previously, we established that purified LipA can hydrolyse acetate, butyrate and caprylate esters of IPG although without enantioselective preference ($E$ ranging from 1.2 to 1.6, data not shown). However, selection of mutants with an improved enantioselectivity requires an enantiopure substrate linked to the inhibitor. This was achieved using an inhibitor which was enantiopure at the chiral atom of the IPG molecule. Although this method requires the synthesis of a chiral enzyme inhibitor, it opens the possibility to evaluate millions of enzyme variants with respect to a given property within a short time.

We showed that LipA expressing phages could bind to the phosphonate suicide inhibitor in the same manner as soluble LipA, since both solutions had similar inactivation patterns with only minor differences in $t_1/2$ values. LipA interacted specifically with the phosphonate moiety of this inhibitor, since a non-immobilised inhibitor inactivated LipA with similar $t_1/2$ values. Since a SIRAN coupled inhibitor containing a C₆ alkyl linkage did not inactivate the phage-bound LipA, the inactivation of LipA is not due to an aspecific interaction with this SIRAN matrix. Further evidence for the specific interaction with the immobilised phosphonate inhibitor is found from the fact that only LipA expressing phages bound to the inhibitor. Correspondingly, M13KO7 helperphages did not bind to the inhibitor, since they could hardly be demonstrated in the eluted fractions (table 2). As a result of this selective binding, the phage mixture can be enriched for almost 100% for LipA expressing phages with an ampicillin resistance gene. The inability to solubilise the SIRAN-immobilised inhibitor allowed us to retrieve the inhibitor with the covalently bound LipA phages from the solution. Afterwards, the covalently bound phages could easily and efficiently be recovered using the collagenase cleavage site. Unfortunately, the insolubility
of the inhibitor prohibited adequate determination of inhibition rate constants for the enzyme-inhibitor-complex.

In conclusion, selective and covalent binding of the phage-bound LipA to a phosphonate suicide inhibitor was demonstrated. This implicates that a system has become available that can be employed for *Bacillus* LipA mutant selection. The synthesis of the *Rc* inhibitor will enable dual selection of mutants with improved enantioselectivity towards IPG.

**Acknowledgements**

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