Enzyme stabilization with solid-phase triazine-scaffolded biomimetic ligands

Thesis to obtain the Master of Science Degree in Biological Engineering

Diogo Faria¹

¹Master Student of Biological Engineering, Instituto Superior Técnico, Lisbon, Portugal

Abstract: Synthetic ligands that mimic the structure and binding of natural biological ligands are designated as biomimetic ligands. After the first uses of textile dyes as pseudo-affinity ligands, this technology started to be the basis of de novo synthesis and rational design concepts of triazine-scaffolded synthetic ligands. This rational approach was formally used to find stabilizing ligands, which could bind cutinase with high affinity while retaining enzymatic activity.

The stabilizing effect on cutinase from Fusarium solani pisi obtained with triazine-scaffolded synthetic ligands, and, particularly with a selected lead-ligand (3’/11), is the basis for future research with triazine-based compounds as probes for enhancing protein stability by adsorption. It is envisaged that some of these ligands are assessed and screened for their potential stabilizing effect on other enzymes.

In the present work, the high affinity of ligand 3’/11 with cutinase was confirmed and the same ligand was screened for its ability to bind with other enzymes, as lipases from Aspergillus oryzae, Candida rugosa, Chromobacterium viscosum, Rhizomucor miehei and Rhizopus niveus, and invertase from Saccharomyces cerevisiae. The enzymes CRL, CVL, RNL and invertase showed significant adsorption yields to ligand 3’/11 – 32, 29, 36 and 94%, respectively –, and the thermostability at 60°C of the free and adsorbed enzymes was studied. CVL and CRL were stabilized by the adsorption to ligand 3’/11. In the cases of CRL and invertase, other ligands, from the original combinatorial library, were tested: CRL was stabilized by adsorption to ligand 3’/3’ and invertase was stabilized by adsorption to ligand 5/3’.

Keywords: Thermostability; affinity interactions; biomimetic ligands; cutinase; lipases; invertase.

INTRODUCTION

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) catalyze the hydrolysis and the synthesis of esters from glycerol and long-chain fatty acids (Sharma et al., 2001). This kind of enzymes is reported to be monomeric, having a molecular weight in the range of 19-60 kDa and, in the addition to its hydrolytic activity, lipases can also catalyze esterification, interesterification, acidolysis, alcoholysis and aminolysis reactions (Hasan et al., 2009).

The lipases used in this study were from Aspergillus oryzae (AOL), Candida rugosa (CRL), Rhizomucor miehei (RML), Rhizopus niveus (RNL) and Chromobacterium viscosum (CVL).

Lipase from A. oryzae was fully characterized with a molecular mass of 41 kDa and it seemed to be a monomeric enzyme (Toida et al., 1995); lipase from C. rugosa is a single polypeptide chain consisting of 543 amino acid and an apparent molecular weight of 60 kDa (Benjamin & Pandev, 1998); lipase from R. miehei has an expected molecular weight between 30 and 32 kDa, being composed of 269 amino acids (Noel & Combes, 2002; Wu et al., 1996); lipase from C. viscosum is an enzyme with 319 amino acid residues and a molecular mass of about 33 kDa – an amino acid sequence identical to that of the lipase from Pseudomonas glumae – with an isoelectric point estimated between 6.9 and 7.1 (Taipa et al., 1992; Taipa et al., 1995; Lang et al., 1996); lipase from R. niveus used in the present work are not fully characterized.

Cutinase (EC 3.1.1.74) from Fusarium solani pisi is a lipolytic enzyme that catalyzes the hydrolysis of cutin. Additionally, cutinase is capable to hydrolyze a vast variety of esters, extending from p-nitrophenyl esters to insoluble long-chain triglycerides (Lauwereys et al., 1991). Cutinase is a 197-residue protein with a molecular weight of 22 kDa (Longhi et al., 1997) and an isoelectric point of 7.8 (Petersen et al., 2001).

External invertase (β-fructofuranosid fructohydrolase, EC 3.2.1.26) from Saccharomyces cerevisiae is an important enzyme in the food industry. This enzyme catalyzes the hydrolysis of sucrose into an equimolar mixture of glucose and fructose, known as inverted sugars (Danicman et al., 2004). The main structure of the external invertase from S. cerevisiae is a homodimer with a molecular weight of 270 kDa and an isoelectric point between 3.4 and 4.4 (Neumann & Lampen, 1967).

Affinity ligands for protein purification can be natural molecules such as enzymes substrates and inhibitors, effectors, coenzymes, hormones, antigens, nucleic acids, and sugars, presenting high-affinity constants and selectivity for their complementary proteins (Lowe et al., 1992). However, the increasing demands for highly purified proteins favored the development of synthetic affinity ligands, generally regarded as safe and more economic alternatives, comparing with natural ligands, that are usually unstable, expensive and often immunogenic, which becomes problematic in case of leakage when purifying protein for use as therapeutics (Roque et al., 2007).

Synthetic affinity ligands are chemically synthesized low-molecular-weight compounds that interact with the target protein by affinity-like interactions (Taipa, 2014). This type of ligands generally shows moderate affinity for the target protein, which can be advantageous since it allows the
employment of mild elution conditions (Sousa & Taipa, 2014). The main advantages of the synthetic ligand for affinity processes are their scalability, durability, capability of reuseage over multiple cycle and low price. They are also commonly not toxic and their exceptional stability enables the harsh elution as well as in-place cleaning and sterilization procedures (Lowe et al., 2001).

The ease of manipulation of the triazine scaffold and the reactivity of cyanuric acid towards amines together with its structural rigidity has attracted a large interest in exploring its combinatorial derivatization for biological applications (Lwik & Lowe, 2000). Several biomimetic triazine-based ligands have been designed as stable synthetic analogues that replaced natural biological ligands (Sousa & Taipa, 2014).

A ligand library had been designed by Roque et al., to select ligands that mimicked protein L from Pectrostrepotocus magnus for the affinity purification of antibodies and small fragments (Roque et al., 2005b).

From the ligands of this library that bound more than 50% of the loaded enzyme, when screened by a chromatography assay, amines 3, 5, 7, 8 and 11 gave generally good results. Amines 1, 2, 4, 6, 9 and 10 reported good binding results usually when combined with one of the aforementioned amines (Sousa, 2010). Thus, the amines chosen for the synthesis of a second-generation ligand library were 3, 5, 7, 8 and 11, combined with amines 3 and 4 of the rational library reported by Ruui et al., 2006, which were after named 3’ and 4’, respectively (Sousa, 2010). This approach used by Ruui et al., 2006 had as rationale the attempt to find stabilizing ligands, which were able to bind cutinase with high affinity while retaining enzymatic activity. Therefore, the target surface regions, or weak regions, were considered suitable for the design of complementary ligands, since these regions were found to be involved in the early unfolding events of cutinase.

The ligands with a combination between one of the amines 3, 5, 7 and 11, with a hydrophobic moiety, and 3’ and 4’, bearing an acidic group, showed a thermostabilizing effect on adsorbed cutinase, but different deactivation profiles were available unless otherwise stated. Sepharose CL-6B was from Sigma-Aldrich. Ligands 3’/3’ and 5’/3’ were previously synthesized by Isabel Teixeira de Sousa.

**Enzymes**

Recombinant cutinase, from Fusarium solani pisi, cloned in pMa/c-CUF plasmid was produced and purified in our laboratory. Lipases from Aspergillus oryzae (AOL), Candida rugosa (CRL), Rhizomucor miehei (RML) and Rhizopus niveus (RNL) were from Sigma-Aldrich. Lipase from Chromobacterium viscosum (CVL) was from Toyo Jozo Enzymes. Pierce bovine serum albumin (BSA) standard solutions were from Thermo Scientific.

**Instrumentation**

SDS-PAGE electrophoresis was performed in a Min-PROTEAN TetraSystem cell from Bio-Rad with power supply apparatus Power Pac 300 from Bio-Rad. In the synthesis of ligands, steps performed at 83°C were carried out in a hybridization oven/shaker from Amershams Pharmacia Biotech. Microplate mixer/incubator was either Titramax 1000 from Heidolph Instruments or Agitator 200 from Aralab. Spectrophotometric readings performed in 1 cm pathlength cuvettes – Hellma® adsorption standard cells – were done on Hitachi U-2000 spectrophotometer. For activity measurements, an electronic stirrer Model 300 from Rank Brothers Ltd was adapted on the cuvette holder, and a Thermomix® MM thermocouple from B. Braun with a thermocouple was used to measure the temperature. Spectrophotometric readings performed in microplates were taken on a SpectroMax384 Plus microplate reader from Molecular Devices.

**Methods**

**Triazine-based ligand synthesis and support derivatizations**

**Epoxy activation of Sepharose CL-6B**

The epoxy activation of Sepharose CL-6B was performed according to a method described by Filippusson et al., 2000.

**Amination of epoxy-activated Sepharose CL-6B**

Epoxy-activated agarose was aminated with ammonia according to a protocol adapted from Roque et al., 2005b. The washed epoxy-activated gel was suspended in 1.5 mL of ammonia per gram of moist gel. The slurry was incubated overnight at 30°C with gentle agitation in a rotary shaker. The aminated support was washed utterly in a sintered funnel with distilled water to remove any traces of ammonia. Washing was carried out until the pH of the washing solution was lowered to the pH of the distilled water and no odor could be detected. Aminated supports were either used immediately and activated with cyanuric chloride or stored in 20% (v/v) ethanol between 0 and 4°C.

**Activation of aminated Sepharose with cyanuric chloride**

Preparation of dichlorotriazinyl gel was performed according to a method adapted from the literature (Palanisamy et al., 1999; Teng et al., 1999; Roque et al., 2005b). Aminated agarose was suspended in a solution acetone/water 50% (v/v) – 1 mL per gram of gel. The slurry was maintained
at 0 °C in an ice bath on a shaker. An amount corresponding to 5 molar equivalent of cyanuric chloride – relative to the extent of amination (≈20 μmol amine groups/g gel) – was dissolved in 8.6 mL of acetone per gram of cyanuric chloride and divided into four aliquots. Each aliquot was added to the aminated gel with intervals of about 30 minutes, maintaining the mixture at 0 °C with constant shaking. The pH was monitored with the aid of pH-indicator strips and maintained neutral by addition of a NaOH 1M solution. The gel was then washed with 2 x 10 gel volumes of each acetone/distilled water mixture (v/v) – 1:1, 1.3, 0:1, 1:1, 3:1, 1.0 – and then with abundant water to remove unreacted cyanuric chloride (Roque, 2004). The cyanuric chloride activated gel was not stored but immediately used for the substitution of R1.

Nucleophilic substitution of the second and third chlorine atoms of dichlorotriazinyl Sepharose

After the activation of aminated Sepharose with cyanuric chloride – previous point – the second and third chlorines – in the R1 and R2 positions, respectively – of dichlorotriazinyl agarose were sequentially substituted. The cyanuric chloride activated gel was divided into aliquots. Each aliquot was used for the substitution of the second available chlorine in the triazine ring with two different aminated compounds: ammonia – amine 0 – or 4–aminobenzoic acid – amine 3’. An amount corresponding to 2 molar equivalents of each amine – relative to the determined density of amine groups in the support (≈20 μmol/g gel) – was dissolved in distilled water. For amine 3’, one equivalent of sodium bicarbonate was also added. The volume of solvent used was 1 mL per gram of gel. Each aliquot of dichlorotriazinyl agarose with respective amine solution was incubated at 30 °C for 24h in a rotary shaker. After this, each gel was thoroughly washed with distilled water on a sintered funnel (Roque, 2004). The R1 monosubstituted ligands were used immediately for the substitution with amino compounds R2.

Enzyme activity assays

Activity towards p-NPA: For AOL, cutinase and CVL, the activity assays were performed in 20 mM Tris-HCl buffer at pH 7.0 or 8.0, at either 30 or 37°C in a stirred cuvette, with a total reaction volume of 1.5 mL. AOL sample was added to attain a final concentration of 14 μg/mL of total protein; cutinase and CVL samples – either a solution of free enzyme or a suspension of derivatized resin with adsorbed enzyme – were added to reach a final concentration of 16 nM and in the case of RML this value was 22 μg/mL. The reaction was initiated by the addition of 15 μL of stock substrate solution. The release of p-nitrophenol was monitored by reading absorbance at 400 nm (ε = 15400 M⁻¹cm⁻¹), every 6 seconds, for one minute (Gonçalves et al., 2000).

Activity towards p-NPB: For RNL, activity assays were performed in 20 mM Tris-HCl buffer at pH 7.0 or 8.0, at either 30 or 37°C in a stirred cuvette, with a total reaction volume of 1.5 mL. RNL sample was added to a final concentration of 6.4 μM, when the case in study was the free enzyme, and 4 μM, when the adsorbed enzyme to the derivatized resin was studied. The release of p-nitrophenol was monitored by reading absorbance at 400 nm (ε = 15400 M⁻¹cm⁻¹), every 6 seconds, for two minutes – in the case of free enzyme – or for five minutes – in the case of the adsorbed enzyme to the ligand.

Activity towards p-NPP: For CRL, enzyme activity was determined by a spectrophotometric assay adapted from Vorderwülbecke et al., 1990.

One enzyme activity unit (U) in the previous assays conditions was defined as the quantity of enzyme that catalyzes the formation of 1 μmol of p-nitrophenol per minute.

Activity using DNS acid method: For invertase, the enzyme activity was measured in a solution with 980 μL of sucrose 5% (v/v) in 20 mM acetate buffer at pH 5.3 and 20 μL of invertase solution, either of free enzyme or of a suspension of derivatized resin with adsorbed enzyme. The solution was incubated at 50°C. After this, 20 μL of this solution were added to 80 μL of MilliQ water and 100 μL of DNS reagent, and it was incubated at 100°C for 5 minutes. After cooling, 500 μL of MilliQ water were added, the solution was shaken and the absorbance read at 550 nm with a spectrophotometer. A standard curve with glucose in a suitable concentration range was used in the determination of the activity. One enzyme activity unit (U) was defined as the quantity of enzyme that catalyzes the hydrolyze of 1 μmol of sucrose to inverted sugar per minute (Nunes et al., 2010).

Screening of enzymes binding ligand 3’/11 by affinity chromatography

Affinity chromatographic assays were performed at room temperature as described previously in (Sousa et al., 2013). Resins were packed into 4 mL columns – 1 mL of packed gel – and were washed with 3x2 mL regeneration solution – 0.1 M NaOH in 30% (v/v) isopropanol –, then with water to bring the pH to neutral and finally equilibration buffer – 20 mM Tris-HCl, pH 8.0 or 20 mM acetate, pH 5.3 in the case of Invertase. For each resin, 1 mL of enzyme solution – 1 mg/mL in 20 mM Tris-HCl, pH 8.0 – was loaded onto the column. Washing with equilibration buffer proceeded and 1 mL fractions were collected until the absorbance at 280 nm became lower than 0.005. A standard curve with bovine serum albumin (BSA) in a suitable concentration range – 0.025 to 2 mg/mL – was used for the determination of total protein. Finally, the columns were regenerated with regeneration solution, followed by distilled water, and stored between 0 and 4°C in 20% (v/v) ethanol.

Standard adsorption assay

The standard adsorption assay was made in the same way described above, but using a different volume of moist gel. The loaded volume and the concentration of enzyme solutions were also different for each case and all these values are presented in the Table 1.

Table 1 – The volume of moist gel, loaded volume and the concentration of enzyme solution for each case study.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Volume of moist gel (mL)</th>
<th>Loaded volume (mL)</th>
<th>Enzyme concentration (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRL</td>
<td>0.50</td>
<td>3.0</td>
<td>1.5</td>
</tr>
<tr>
<td>Cutinase</td>
<td>0.50</td>
<td>1.0</td>
<td>0.50</td>
</tr>
<tr>
<td>CVL</td>
<td>0.75</td>
<td>5.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Invertase</td>
<td>0.75</td>
<td>1.0</td>
<td>0.75</td>
</tr>
<tr>
<td>RNL</td>
<td>0.75</td>
<td>5.0</td>
<td>4.5</td>
</tr>
</tbody>
</table>
The resins were removed from the column, suspended in 1 mL of 20 mM Tris-HCl, pH 8.0 – or 20 mM acetate, pH 5.3 in the case of invertase – and then centrifuged at 11500 rpm for 5 minutes allowing the complete settling before the enzymatic thermostability assay.

Thermostability of free and immobilized enzymes

Thermostability assays were performed by measuring the irreversible loss of activity upon incubation at 60°C of both free and immobilized enzyme adsorbed. The concentration of free and immobilized enzymes solution with different concentrations are presented in Table 2.

The suspensions of resin – with bound enzyme – were incubated in a dry bath – Accublock™ Digital Dry Bath from Labnet International, Lda – at 60ºC. Samples were taken at several times of incubation and added directly to an Eppendorf already containing a solution of 20 mM Tris-HCl buffer, pH 8.0 or 20 mM acetate buffer, pH 5.3, in the case of invertase. The sample volumes and the spectrophotometric method used were different for each enzyme: in CRL case, 40 μL and the enzyme activity was measured using p-NPP as substrate; for cutinase and CVL, samples of 15 μL were taken and the activities were measured using p-NPA as substrate; for RNL, samples of 100 μL were taken and the activities were measured towards p-NPB; for invertase, the method used was the same described in using DNS acid method.

Table 2 – Concentration of the solutions of free enzymes and suspensions of immobilized enzymes studied.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Free enzyme concentration (mg/mL)</th>
<th>Immobilized enzyme concentration (mg/mL solution)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRL</td>
<td>3.5</td>
<td>2.0 x 10^-1</td>
</tr>
<tr>
<td>Cutinase</td>
<td>3.5 x 10^-2</td>
<td>3.5 x 10^-2</td>
</tr>
<tr>
<td>CVL</td>
<td>5.2 x 10^-2</td>
<td>1.6 x 10^-1</td>
</tr>
<tr>
<td>Invertase</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>RNL</td>
<td>8.0</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Protein determination by the BCA assay

A microplate assay was used to determine protein concentrations by the BCA method (Smith et al., 1985) according to the instructions in the BCA™ Protein Assay kit (suitable for concentrations ranging from 20 to 2000 μg/mL) or the Micro BCA™ Protein Assay kit (for concentrations between 2 and 40 μg/mL) from ThermoScientific.

SDS-PAGE

The cassettes – Bio-Rad (Hercules, CA, USA) – containing the resolving and stacking gels were assembled in the electrophoresis tank and the chamber properly filled with running buffer (192 mM glycine, 25 mM Tris, and 0.10% (w/v) SDS, pH 8.3). Samples preparation was accomplished by addition of 25 μL of Laemmli buffer from Bio-Rad (277.8 mM Tris-HCl pH 6.8, 4.4% LDS, 44.4% (w/v) glycerol, 0.02% bromophenol blue) and 5 μL of 1M DTT, into 20 μL of sample solutions. Then the solutions were boiled for 10 minutes in water at 90°C and loaded into each well. It was also loaded into one well the molecular weight marker used (SDS-PAGE molecular weight (MW) markers were Precision Plus Protein™ Dual Color Standards (10–250 kDa) from Bio–Rad). Electrophoresis at 90V for 2 hours took place in the next step, being the run stopped when the bromophenol blue reached the bottom of the gel. After this step, the gel apparatus was dismantled, the resolving gel was carefully removed from the gel plates and placed in a staining solution. The gels were stained with a silver-staining method.

The gels were placed in the fixation solution (30% ethanol, 10% acetic acid) for, at least, 2 hours and incubated at 20°C in a rotary shaker (60 rpm). Then the gels were washed with ethanol 30% for, at least, 10 minutes. After this, the gels were washed twice, 10 minutes each, with MilliQ water. The next step was the incubation with the oxidizer solution (0.8 mM sodium thiosulfate) for 1 minute. After this incubation, the gels were washed three times with MilliQ water, 30 seconds washing each. Then, the gels were stained with fresh 8.85 mM silver nitrate solution for 30 minutes. After this step, the gels were washed with MilliQ water for 1 minute, following by incubation with developer solution (0.283 M sodium carbonate, 0.05% (v/v) formaldehyde). The last step consisted in stopping the developing state by immersion of the gels in 5% (v/v) acetic acid solution.

RESULTS

Characterization of cutinase and lipases

Cutinase from Fusarium solani pisi and Lipases from Aspergillus oryzae (AOL), Candida rugosa (CRL), Chromobacterium viscosum (CVL), Rhizomucor miehei (RML) and Rhizopus niveus (RNL) were fully characterized in previous works (Longhi et al., 1997; Toida et al., 1995; Benjamin & Pandev, 1998; Taipa et al., 1992; Noel & Combes, 2002; Kohno et al., 1994). However, given the objectives of the present work, it was necessary to verify some properties as their molecular weight, purity degree and enzymatic activity.

SDS-PAGE analysis

An analysis by SDS-PAGE was performed in order to evaluate the molecular weight and the purity degree of the enzymes to be studied (Figure 1). Recombinant cutinase (lane 2) was totally pure and the molecular weight obtained for this protein was 23 kDa, a value identical to others previously presented (Emmond & Vlieg (2000); Longhi & Cambillau (1999); Martinez et al., 1992). In the case of Chromobacterium viscosum lipase (Figure 1, lane 3), the major band appears at 33 kDa, as expected (Taipa et al., 1992; Taipa et al., 1995; Lang et al., 1996). This enzyme preparation showed a very slight degradation – bands of low molecular weight –, probably result of unspecific proteolytic digestion during the purification and/or storage (Sousa, 2010). The solution of Aspergillus oryzae lipase (lane 4) presented a myriad of proteins. Analyzing the gel, two thick bands appear, approximately, at 50 kDa. Knowing that the described molecular weight of AQL is 41 kDa (Toida et al., 1995), the band that appears immediately below 50 kDa can be this lipase. Rhizomucor miehei lipase is analyzed in lane 5, presenting several bands. As well, the first thing that should be noted is the band located at 31 kDa, value in the range of molecular weights described for this lipase (Wu et al., 1996). Lane 6 shows also a relatively impure extract. The strongest band, located at 81 kDa, seems to be the lipase from Rhizopus niveus, enzyme from Sigma with a calculated molecular weight of 83 kDa. However, one of the most known lipases from Rhizopus niveus has a molecular mass of 30 kDa (Kohno et al., 1994).
et al., 1994) and, bearing in mind this fact, the band located at 31 kDa can also represent this enzyme. The Candida rugosa lipase extract from Sigma was also analyzed. Despite the bad quality of the staining in this lane, it is clear that the most solid band, appearing at 63 kDa, is possibly the lipase itself with a documented molecular weight of 60 kDa (Benjamin & Pandev, 1998).

Figure 1 – SDS-PAGE analysis of the different enzymes under study. Lane 1: Molecular Weight Markers; lane 2: cutinase (10 μg of total protein); lane 3: CVL (10 μg of total protein); lane 4: AOL (30 μg of total protein); lane 5: RML (30 μg of total protein); lane 6: RNL (30 μg of total protein); lane 7: CRL (35 μg of total protein).

Enzymatic activities

The characterization of enzymatic activities is important in order to evaluate the optimal pH and temperature to make the comparisons between free and adsorbed enzymes. Firstly, it must be noted that only four pairs of conditions with two different temperatures, 30 and 37 °C, and two distinct pH, 7.0 and 8.0, were tested for all enzymes. The choice of these restricted conditions was based upon the fact that most of the lipases are stable and have the optimal activity between 6 and 7.5 (Ghosh et al., 1996), regarding the optimal pH, and between 30 and 50 °C, in the case of temperature. However, above 40 °C, the stability of many lipases is affected, circumstance that may change the protein conformations and, consequently, the enzyme activity measurements. The optimal conditions obtained were used in the activity measurements over the present work, except in the case of cutinase, for which the temperature used was 30 °C.

Table 3 – Optimal pH and temperature, and specific activity of cutinase and different lipases.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Optimal pH</th>
<th>Optimal Temperature</th>
<th>Specific Activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOL</td>
<td>8.0</td>
<td>37</td>
<td>3.4 ± 0.5</td>
</tr>
<tr>
<td>CRL</td>
<td>8.0</td>
<td>37</td>
<td>0.71 ± 0.7</td>
</tr>
<tr>
<td>Cutinase</td>
<td>7.0</td>
<td>30</td>
<td>160 ± 70</td>
</tr>
<tr>
<td>CVL</td>
<td>7.0</td>
<td>30</td>
<td>98.5 ± 0.9</td>
</tr>
<tr>
<td>RML</td>
<td>7.0</td>
<td>37</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td>RNL</td>
<td>8.0</td>
<td>37</td>
<td>(3.52 ± 0.08) x 10⁻²</td>
</tr>
</tbody>
</table>

Screening of lipases binding to ligand 3'/11 by affinity chromatography

The screening of lipases binding to ligand 3'/11 by affinity chromatography was done. This synthetic affinity ligand was previously proven to bind cutinase with high affinity while preserving its biological activity (Sousa et al., 2009) and to stabilize bound cutinase at elevated temperatures (Sousa et al., 2013, Figure 2).

Figure 2 – Structure of ligand 3'/11, comprising 4-aminobenzoic acid as substituent in R1 position (mimic of Aspartic and Glutamic acids) and 2-methylbutylamine in R2 position (mimic of Isoleucine).

The lipases that consistently gave good results in terms of binding to this support were selected to perform thermostability assays. Adsorptions to Sepharose CL-6B, dichlorotriazinyl gel and to ligand 0/0 were also done and used as controls, to evaluate whether these supports could bind the enzymes by non-specific interactions.

Within the group of enzymes studied in this work, two of them (A. oryzae and R. miehei lipases) showed very low adsorption yields – (13 ± 2) and (11 ± 2)%, respectively, to ligand 3'/11 – and non-specific interactions were not found in the assays with the three control supports (data not shown). Due to the low values of adsorption yields, these two lipases were left behind and no further studies were done in terms of stabilization of the adsorbed enzymes to this ligand.

Figure 3 summarizes the adsorption yields of cutinase, and of lipases from C. viscosum, R. niveus and C. rugosa to the ligand 3'/11, Sepharose CL-6B, dichlorotriazinyl gel and to ligand 0/0.

Figure 3 – Protein adsorption yields obtained for all the enzymes in the assays with ligand 3'/11 (orange), Sepharose CL-6B (blue), dichlorotriazinyl gel (green) and ligand 0/0 (grey).

Cutinase presented the higher adsorption yield and its value was very close to the one reported by Sousa et al., 2009, 79%. Regarding the other enzymes, it should be noted that lipases from R. niveus and from C. rugosa showed higher adsorption yields when comparing with lipase from C. viscosum, which made them good candidates to the thermostability assays with this ligand.

The data in Figure 3 also shows that the adsorption yields obtained in the assays with the dichlorotriazinyl gel (between 15 and 30%) are not negligible. Higher adsorption to this control-gel can be explained by the high reactivity of the two
existential chlorine atoms and the possible reaction with the bonded enzymes. In order to surmount this effect, these assays were performed at low temperature (4 °C) with the purpose of decreasing the reactivity of the chlorine atoms that is favored at higher temperatures (Unsworth et al., 2007). However, regarding the adsorption observed to ligand 0/0, it can be seen that for all enzymes, except for lipase from C. rugosa, the adsorption yields obtained in the assays with this ligand are negligible. The difference between this support and the dichlorotriazine gel is the substitution of two chlorine atoms by two amine groups, therefore, these results suggest that the triazine ring is not responsible for any kind of interactions with most of the enzymes in question. In the case of CRL, at pH 8.0, this lipase is negatively charged, because its pI of 4.5 (Petersen et al., 2001) while almost all diaminetriazinyl groups of this support are neutrally charged, since its pKa is around 5.0 (Jang et al., 2009), i.e., the high adsorption yield observed is probably not due to ionic interactions with ligand 0/0, but likely to other type of non-specific interaction.

The degree of immobilization of each enzyme to ligand 3′/11 was also studied using the adsorption yield in terms of enzymatic activity – \( \eta_{act} \) – given by the Equation 1.

\[
\eta_{act}(\%) = \left(1 - \frac{\text{Act}_i}{\text{Act}_0}\right) \times 100 \quad \text{Eq.1}
\]

Where Act\(_i\) is the total activity of fraction number \( i \), in U, and Act\(_0\) is the total activity of the initial enzyme solution, also in U. The \( \eta_{act} \) values obtained for all enzymes are presented in the Table 4.

In the case of cutinase, the yield obtained was expectable given the high purity of the enzyme used previously as discussed and the results previously reported by Sousa et al., 2009, 2013. The \( \eta_{act} \) of lipase from C. viscosum was almost half of the protein adsorption yield obtained with this lipase – vide Table 4. This fact can be explained by two reasons: the proteins of low molecular weight included in CVL powder (vide Figure 1) were adsorbed, increasing the value of protein adsorption yield, since the quantification in this case is made using the absorbance at 280 nm, and this method does not distinguish different proteins/peptides; and/or the process of loading and washing could be affecting the lipase thus diminishing its activity and the value of \( \eta_{act} \).

### Table 4 – Comparison of protein (\( \eta_{prot} \)) and activity (\( \eta_{act} \)) adsorption yields to ligand 3′/11 obtained for different lipolytic enzymes. The results were obtained in triplicates.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>( \eta_{prot} ) (%)</th>
<th>( \eta_{act} ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cutinase</td>
<td>75 ± 1</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>CVL</td>
<td>29 ± 3</td>
<td>14.7 ± 0.6</td>
</tr>
<tr>
<td>CRL</td>
<td>36 ± 5</td>
<td>70.9 ± 0.6</td>
</tr>
<tr>
<td>RNL</td>
<td>32 ± 5</td>
<td>100 ± 0</td>
</tr>
</tbody>
</table>

In the case of lipase from C. rugosa, the \( \eta_{act} \) is more than two times higher than the adsorption yield relative to this lipase (vide Table 4). In this case, the preferential adsorption of the proteins of low molecular weight is the most likely explanation. A SDS-PAGE analysis of the fractions collected in the CRL assay was performed and it was confirmed that around 30% did not adsorb to the ligand (data not shown), result that is in accordance with the \( \eta_{act} \) to this lipase.

The \( \eta_{act} \) obtained for R. niveus lipase can be erroneous, given that the measuring of the total activity of this lipase is only possible for concentrations above 5.0 µM or 0.42 mg/mL and not all fractions collected presented concentration above this value (data not show). Therefore, a SDS-PAGE analysis of the fractions collected in the RNL assay was done and it was confirmed that this lipase was totally adsorbed to ligand 3′/11 (data not shown).

### Thermostability of free and adsorbed lipases to ligand 3′/11

The thermostability of the enzymes that showed significant adsorption to ligand 3′/11 – cutinase, and lipases from C. viscosum, C. rugosa and R. niveus – was studied by comparing the behavior of the free and adsorbed enzymes, to verify if the interactions with this support increased the stability of the proteins at a temperature of 60°C.

### Thermostability assays with cutinase

The results of thermostability assays with cutinase are presented in the Figure 4.

![Figure 4](image-url)

**Figure 4 – Deactivation of cutinase free (blue) and adsorbed to ligand 3′/11 (orange) at 60 °C. The incubation was performed in 20 mM Tris-HCl, pH 8.0, and the concentration of enzyme was 35 µg/mL solution in both cases.**

The data on Figure 4 shows that the deactivation of free cutinase is explained by a first order law, typical kinetics of free enzymes (Gama et al., 2003). The half-life time (t(1/2)) obtained was around 50 minutes, a value slightly higher than the one reported by Sousa et al., 2013 – 35 minutes –, using the same pH and temperature. However other conditions as agitation or the cuvette material may have been different, explaining the difference between the two values.

In the case of adsorbed cutinase, Figure 4 shows that its deactivation can be fitted by a four-parameter biexponential model of Henley and Sadana (Henley & Sadana, 1985) The values of the coefficients \( \beta_1, \beta_2, k_1 \) and \( k_2 \) obtained were 1.00, 0.00, 5.00 x 10⁻¹ min⁻¹ and 8.02 x 10⁻⁵ min⁻¹, respectively. These values are typical of a convex biphasic deactivation as it can be seen in Figure 4.

Comparing the deactivation profile obtained in this study with that achieved in Sousa et al., 2013, it is possible to verify that the two are slightly different. While in the present work there is an initial significant decrease in the relative enzyme activity, in the data reported by Sousa, 2010 this reduction was not observed. However, in both cases, it was seen that the activity of the adsorbed cutinase to ligand 3′/11 remained almost constant during the time of incubation (Sousa et al., 2013).

Although the specific activity relative to the adsorbed cutinase in this work – 105 U/mg – was very different of the value reported by Sousa (2010) – 50 U/mg –, after 90 minutes of incubation at 60°C, the residual activity of adsorbed Cutinase...
shown was very similar in the two cases – 87% and 94%, respectively (Sousa et al., 2013).

**Thermostability assays with CVL**

The results of thermostability assays with CVL adsorbed to ligand 3'/11 are presented in the Figure 5.

![Figure 5](image-url)

**Figure 5** – Deactivation of lipase from *C. viscosum* free (blue) and adsorbed to ligand 3'/11 (orange) at 60°C. The incubation was performed in 20 mM Tris-HCl, pH 8.0, and the concentrations used were 52 µg/mL and 160 µg/mL solution for free and adsorbed CVL, respectively.

Analyzing Figure 5, it is shown that the deactivation of free lipase from *C. viscosum* was explained by a first order law, a typical kinetics of free enzymes (Gama et al., 2003). The half-life time (t<sub>1/2</sub>) obtained was 39 minutes, value very similar to the one reported by Castellar et al., 1997 – 45 minutes. The difference can be explained by the pH assay of 9.0 by Castellar et al., 1997, different from the pH value of 8.0 used in this work.

The profile obtained for *C. viscosum* lipase adsorbed to ligand 3'/11 (Figure 5) was similar to that previously described by Sousa, 2010 and it could be fitted by a four-parameter biexponential model of Aymard and Belarbi (Aymard & Belarbi, 2000). The values of the coefficients A, B, α and β obtained were 0.58, 0.42, 1.0 x 10<sup>-3</sup> min<sup>-1</sup> and 2.56 x10<sup>-2</sup> min<sup>-1</sup>, respectively.

Additionally, it should be mentioned that the high dispersion and the lower number of points in the adsorbed enzyme assay resulted from inherent errors caused by the resin in the spectrophotometric method and by the reduction of assay volume due to sampling. As so, though stabilization occurred, it was not possible to prove that this stabilization lasts at least 3 hours as reported by Sousa (2010).

Finally, the percentage of specific activity of adsorbed CVL relative to the free lipase is around 8%, which is in compliance with a typical reduction in immobilized enzymes activity (Gama et al., 2003).

**Thermostability assays with CRL**

It was observed that the deactivation of free lipase from *C. rugosa* is explained by a first order law, similar to that presented by lipase from *C. viscosum*. The half-life time obtained was 35 minutes and within 60 minutes, the lipase presented 30% of residual activity, in contrast to the value of 10% reported by Soares et al., 1999. The difference between the two results can be explained by the slightly different conditions used in the assays – 20 mM Tris-HCl, pH 8.0, in the present work and 100 mM phosphate, pH 7.5, in the study of Soares et al., 1999.

The deactivation of lipase from *C. rugosa* adsorbed to ligand 3'/11 is explained by the same first order law of the free enzyme (data not shown). The similar deactivation profiles of free and bound enzyme could also be explained if the adsorbed lipase was released to the buffer during incubation time. Though it was proven that this did not occur for cutinase bound to ligand 3'/11 (Sousa et al., 2013), a control of the protein in solution was performed, but the results were not conclusive.

The percentage of specific activity of adsorbed CRL relative to the free lipase was around 400% (0.8±0.2 U/mg of free lipase vs 2.8±0.8 U/mg of adsorbed enzyme) result that can indicate that free CRL might be aggregated in solution, which makes its measured activity (vide Table 3) lower than the real one.

**Thermostability assays with RNL**

The results obtained in the thermostability assays with lipase from *R. niveus* adsorbed to ligand 3'/11 are presented in Figure 6.

![Figure 6](image-url)

**Figure 6** – Deactivation of free RNL (blue) and RNL adsorbed to ligand 3'/11 (orange) at 60°C. The incubation was performed in 20 mM Tris-HCl, pH 8.0, and the concentrations used were 8 mg/mL and 5 mg/mL solution for free and adsorbed RNL, respectively.

The deactivation of the free lipase, in this case lipase from *R. niveus*, was also explained by a first order kinetic law, with a half-life time of 3.5 minutes, value that cannot be directly compared with other reported in the literature by Kohno et al., 1994, 2000, since these results are referred to other lipases of the same organism.

The case of lipase from *R. niveus* was a specific one in the group of lipases studied. Analyzing Figure 6, the stabilization occurred did not coincide with the cases reported previously for cutinase (vide Figure 4) and for lipase from *C. viscosum* (vide Figure 5), because for RNL, the immobilized lipase has a deactivation explained by a first order kinetic law, presenting a half-life time of 11 minutes, three times higher than that relative to the free enzyme. However, the enzymatic activity of bound enzyme did not remain constant for a long period, as it happened in the cases of the other two enzymes mentioned above.

Lastly, it should be mentioned that the percentage of specific activity of adsorbed lipase from *R. niveus* relative to the free lipase was around 52% (48±9 U/mg of free lipase vs 25±6 U/mg of adsorbed enzyme), value that proves, in certain way, that RNL was indeed immobilized, since adsorbed enzymes have, in general, lower specific activities relatively to the free enzymes in solution (Gama et al., 2003). However, the measurement of the quantity of protein in solution during the assay was not conclusive, which did not allow the verification that the enzyme was not eluted from the support during the thermostability assay.
Thermostability assays with CRL adsorbed to a different ligand

The deactivation profiles of lipase from *Candida rugosa* free and adsorbed to ligand 3'/11 had the same behavior. Therefore, it was envisaged to choose amongst other available ligands of the second-generation combinatorial library, generated to bind and stabilize cutinase in other to study the adsorption and the potential stabilization of the lipase from *C. rugosa*.

Selection of the ligands

The ligands selected as alternative to 3'/11 were: i) mixed ligands, containing both a hydrophobic and an acidic group that were previously reported to display a stabilizing effect on cutinase; ii) among the mixed, those binding less cutinase and with lower activity after binding (Sousa, 2010; Sousa et al., 2009, 2013). Following these criteria, two ligands were selected designated as 3'/3' and 5/3', respectively (Figure 7).

Adsorption of CRL to ligands 3'/3' and 5/3'

![Structure of ligands alternatives to 3'/11 used in the study of thermostability of adsorbed CRL](image)

Figure 7 – Structure of ligands alternatives to 3'/11 used in the study of thermostability of adsorbed CRL. A: ligand 3'/3' comprises tyramine as substituent in R₁ position (mimic of Tyrosine) and 4-aminobenzoic acid in R₂ position (mimic of Aspartic and Glutamic acids); B: 5/3' comprises isoamylamine as substituent in R₁ position (mimic of Leucine) and 4-aminobenzoic acid in R₂ position (mimic of Aspartic and Glutamic acids).

Adsorption yields of CRL to the ligands 3'/11, 3'/3', 5/3', 0/0, Sepharose CL-6B and dichlorotriazynil gel are represented in Figure 8. As it can be observed, statistically, the ligand with the highest adsorption yield of CRL was 3'/3' (support B) while the value relative to 5/3' (support C) was very similar to that obtained to the ligand 3'/11 (support D). From data in Figure 8, it is possible to verify that ligands with only one amine containing an aromatic ring (3'/11 and 5/3') adsorbed a lower mass of lipase from *C. rugosa* than the ligand with two aromatic rings: 3'/3'. However, the adsorption yield obtained with ligand 0/0 was high and comparable to that obtained with ligand 3'/3'. This result (that should be confirmed in further studies) does not allow to conclude that the two phenyl groups are responsible for a considerable specific adsorption of the enzyme. The alkane branches seem to affect negatively the adsorption yields of CRL, given that the presence led to lower yields. The results obtained with ligand 0/0 and the dichlorotriazynil gel seem to indicate that the triazine ring is not innocuous in the adsorption of CRL in contrast to the other enzymes.

Thermostability assays with CRL adsorbed to ligand 3'/3'

The deactivation profile of free lipase from *C. rugosa* at 60 °C is explained by a first order law, typical kinetic of free enzymes (Gama et al., 2003). The half-life time (tᵋ/₂) obtained was 35 minutes. The experimental data for the immobilization enzyme were fitted by the model of Aymard and Belarbi (orange curve) and by the one of Henley and Sadana (green curve); using the method of least squares and the Solver tool of Microsoft Excel. The two deactivations are displayed in Figure 8.

![Deactivation profile of free CRL (blue) and CRL adsorbed to ligand 3'/3' (orange) at 60 °C](image)

Figure 9 – Deactivation profile of free CRL (blue) and CRL adsorbed to ligand 3'/3' (orange) at 60 °C. The orange and green curves are models of Aymard and Belarbi, and Henley and Sadana, respectively. The incubation was performed in 20 mM Tris-HCl, pH 8.0, and the concentrations used were 3.5 mg/mL and 200 μg/mL solution for free and adsorbed CRL, respectively.

The coefficients relative to each model are shown in Table 5. The first model used was the Aymand and Belarbi one (orange curve) due to its more simplistic formalism. Analyzing the data in Table 5, it can be seen that the sum of A and B terms is not equal to one, in contrast to what was observed in the case of *C. viscosum* lipase. This fact indicates that this model is not likely the most correct one to use in this case, since the null value of β allows to verify that the final conformation of CRL, retains some activity (Aymard & Belarbi, 2000). Additionally, observing the orange curve of the Figure 4.13, it is evident that this model did not fit well to the experimental points in the final phase of the deactivation (between 50 and 90 minutes). As so, alternatively, the model of Henley and Sadana was fitted to the experimental data. As it was stated above, β₂ is not only unequal to zero, but also higher than β₁. The value estimated for β₁ was 0.00 but it was verified that values up to 0.4 did not change significantly the error of the model (data not shown); it can also be seen that k₂ is more than 10 times larger than k₁.
Having in consideration the values obtained for the coefficients of the model of Henley and Sadana and its series-type formalism, the difference between the values of $k_1$ and $k_2$ means that the intermediate conformation, $E_i$, is produced 10 times slower than the final specie $E_2$. However, in the first 20-30 minutes of the incubation, it is the intermediate conformation $E_i$ that is exclusively formed and this fact explains the initial deactivation of the relative enzyme activity (vide Figure 9). After 40 minutes of incubation, the final enzyme conformation $E_2$ increases its concentration relative to $E_1$ and the enzyme activity of adsorbed CRL reaches a plateau corresponding to the activity of $E_2$ (different from zero) – Figure 9.

The explanation suggested for the stabilization of the adsorbed cutinase was that the existence of polar and charged residues relatively well distributed on its surface, with non-polar patches also present, might allow the ligands, bearing a combination of moieties with different character, to find appropriate binding complementary sites (Sousa, 2010). Regarding the results obtained for lipase from C. rugosa, this enzyme was stabilized by ligand 3/3’, but the same did not occur with ligand 3/11.

The structure of lipase from C. rugosa was determined and refined at 2.1 Å resolution (Grochulski et al., 1993). This lipase reported two main conformational states in aqueous solution: open and closed. The first represents the active conformation adopted by the enzyme near or at the interface and the last one constitutes the inactive form of the lipase, where the flap (an elongated loop that lies flat on the protein surface above the active site) is tightly fixed by a disulfide bridge and by a salt bridge. The open conformation is a result of the opening of this flap (Grochulski et al., 1993).

The stabilization achieved when C. rugosa lipase was adsorbed to a ligand with phenyl groups in both arms can suggest that the phenylalanines placed in the hydrophobic site of the flap (Grochulski et al., 1993) interact with such groups and play a major role in the attained stabilization of the lipase with the ligand 3/3’. However, this hypothesis can only be confirmed by molecular modelling studies that will unravel the most likely binding sites to both ligands 3/11 and 3/3’.

**Thermostability of invertase adsorbed to different ligands**

**Screening of ligands for binding invertase by affinity chromatography**

Ligands 3/11, 3/3’ and 5/3’ were screened as affinity binders to invertase whereas controls for unspecific binding were carried out with Sepharose CL-6B, dichlorotriazinyl gel and ligand 0/0 (with amines at both R1 and R2 positions). Results are shown in Figure 10.

Among the three ligands, 3/11, 3/3’ and 5/3’, ligand 3/11 presented the higher adsorption yield for invertase. Comparing the results, it is possible to see that the ligands with an amine having a phenyl group in position $R_1$ (3/11 and 3/3’) presented slightly higher adsorption yields for invertase than the ligands with an aliphatic amine (5/3’).

---

**Table 5** – Coefficients obtained to the deactivation of CRL adsorbed to ligand 3/3’, using the models of Aymard and Belarbi, and of Henley and Sadana.

| Ligands | Aymard and Belarbi | | | Henley and Sadana | | |
|---------|-------------------| | | Beta (min⁻¹) | | |
|         | $\alpha$ (min⁻¹) | | | $\beta_1$ | | |
| 3/11    | 0.431             | | | 0.00         | | |
| 3/3’    | 3.56 x 10⁻²       | | | 0.525        | | |
| 0/0     | 0.471             | | | 5.84 x 10⁻²  | | |
|         | 0.00              | | | 0.606        | | |

---

**Figure 10** – Adsorption yields obtained for invertase with ligand-derivatized supports. A: ligand 3/11 (triplicate); B: ligand 3/3’ (triplicate); C: ligand 5/3’ (triplicate); D: ligand 0/0 (control, duplicate); E: Sepharose CL-6B (control, duplicate); F: dichlorotriazinyl gel (control, single assay).

The data from Figure 10 also shows that the Sepharose CL-6B adsorbed a significant amount of invertase loaded onto the column. This result seems to indicate that the adsorption to ligand-derivatized supports might not be entirely specific but also governed by non-specific interactions.

**Thermostability of invertase free and adsorbed to ligand 3/11**

The deactivation of the free enzyme, in this case invertase at 60°C, is explained by a first order deactivation law, with a $t_{1/2}$ of 14 minutes, very similar to the one described by Andjelković et al., 2010 (12 minutes, at 60°C in deionized water). In the present work, invertase, incubated in 20 mM acetate buffer, pH 5.3 at 60°C, after a period of 25 minutes, presented 35% enzyme activity, while Andjelković et al., 2012, reported a value of 20%, in deionized water at 60°C, upon the same time. The difference might be explained having in consideration the solution used in the assay (deionized water) and the fact that proteins can be stabilized by salts in solution (Ugwu & Apte, 2004). The deactivation profile of adsorbed invertase followed also a first order law, very similar to the one of the free enzyme, with a $t_{1/2}$ of 13 minutes, which indicates that no stabilization was achieved with the immobilization of invertase in this support (data not shown).

**Thermostability of invertase free and adsorbed to ligand 5/3’**

After the non-stabilization of invertase adsorbed to ligand 3/11 was shown, a single assay of thermostability of this enzyme immobilized to ligand 5/3’ was done as alternative. Figure 11 shows the deactivation of free and adsorbed invertase to ligand 5/3’ at 60°C. Analyzing Figure 11, it can be seen the deactivation of free invertase, previously described, and the decay of the activity of the enzyme adsorbed to ligand 5/3’ and it can be noted that this ligand was able to stabilize the enzyme. The model of Henley and Sadana was fitted to the experimental data, and the values obtained for coefficient $\beta_1$, $\beta_2$, $k_1$ and $k_2$ were 1.65 x 10⁻¹, 4.45 x 10⁻³, 2.97 x 10⁻¹ min⁻¹ and 1.99 x 10⁻¹ min⁻¹, respectively.

Finally, given that the adsorption of invertase to Sepharose CL-6B was very significant, an additional thermostability
assay was done to verify if this enzyme was stabilized by the immobilization in this support and it was proven that stabilization did not occur when invertase was adsorbed to Sepharose CL-6B (data not shown).

Figure 11 – Deactivation of free invertase (blue) and invertase adsorbed to ligand 5′3′ (orange) at 60 °C. The incubation was performed in 20 mM acetate buffer, pH 5.3, and the concentrations used were 2.0 mg/mL of solution in both cases.

CONCLUSIONS
Overall, this work provided additional proof-of-concept that triazine-scaffolded synthetic affinity ligands, combining a substituent that mimics a hydrophobic amino acid side-chain and another bearing a carbohydrate moiety, when bound to beaded agarose, may bind different proteins and act as thermostabilizers of bound enzymes.

REFERENCES