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ARTICLE

Substrate's hydrophobicity and enzyme's modifiers play a major role on the activity of lipase from *Thermomyces lanuginosus*

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Lipase from *Thermomyces lanuginosus* (TL) displays high affinity for long-chain substrates, such as triolein and other long-chain triacylglycerols. Aiming to broaden the substrate chain-length specificity, different aldehydes (naphtaldehyde, butyraldehyde, hexyl aldehyde and dodecyl aldehyde) and naphthyl isothiocyanate were grafted onto lipase TL through lysine coupling. The catalytic activity of the modified lipases was investigated by reaction with substrates differing in the aliphatic chain size (*p*-nitrophenyl benzoate, *p*-nitrophenyl acetate, *p*-nitrophenyl butyrate, *p*-nitrophenyl hexanoate, *p*-nitrophenyl octanoate, *p*-nitrophenyl laurate and *p*-nitrophenyl palmitate). The enzymes modified with aldehydes revealed higher activity than the enzymes modified with the isothiocyanate. The most notable results were achieved for lipase TL grafted with 4 units of a dodecyl chain (TL5), which revealed the highest activity against all the tested substrates, being 10-fold more active than the native enzyme for smaller substrates (acetate and butyrate chains), and 2-fold for longer (laurate and palmitate chains). The kinetic parameters evaluated (V_{max}, K_M and k_{cat}/K_M) also confirmed the significant catalytic performance of TL5 comparing to the native enzyme. The increase of activity revealed by the modified lipases was directly proportional to the size and hydrophobicity of the linkers' aliphatic chain. Small conformational changes, either on the enzymes' lid as on the cavity of the active site were suggested by Molecular Dynamics simulations, Circular Dichroism and Fluorescence spectroscopy. Moreover, the grafting with aldehydes or with the isothiocyanate, conferred higher thermostability to the lipase. The chemical surface modification developed efficiently improved the activity of lipase TL, broadening the substrates chain-length specificity, incrementing thereafter the substrate possibilities for industrial reactions.

Introduction

Lipase from *Thermomyces lanuginosus* (EC 3.1.1.3) was firstly isolated from compost medium containing, among other components, long-chain esters like soybean oil, corn steep liquor, starch[1]. The natural function of lipase from *Thermomyces lanuginosus* is described in literature as the degradation of the long-chain triacylglycerols present in the compost medium[1-3].

Nowadays, this lipase is produced using a recombinant strain of *Aspergillus oryzae,* a filamentous fungus often used for enzyme production[4], and the isolated enzyme is responsible for the lipolytic activity of Lipolase®, used for many applications like the production of flavours, biodiesel and fine chemicals[5]. It is also commonly used for the hydrolysis of substrates such as triolein[6] and other long-chain triacylglycerols[5, 7]. This enzyme possesses 1,3-stereospecificity, which indicates that it specifically hydrolyses the ester group of triacylglycerols in the position 1 and 3. The hydrolysis of the position 2 occurs by migration of the acyl group to the position 1, and further hydrolysis by the enzyme. The surface modification of enzymes is a strategy often applied to increase their global properties, mainly the thermostability. The immobilization of enzymes onto solid supports, is the strategy mostly studied, with diverse associated advantages, such as easy recovery, increase of resistance and robustness, among others[8].

Lipase from *Thermomyces lanuginosus* is mainly used in its immobilized form, as reported by several authors, due to the higher catalytic performance acquired in the immobilized form, comparing to the free form. Cipolatti and co-workers[9], studied the adsorption of lipase TL onto PEGylated polyurethane particles, and confirmed the improvement in the production of ethyl esters comparing to the free native enzyme. Vasconcellos *et al*[10], reported the immobilization of lipase TL on nanozeolites. They observed an increase on the production yield of biodiesel using these novel nanozeolite-enzyme complex.

Despite the promising results reported so far, the use of solid supports for enzyme immobilization often reduces its catalytic activity resulting from the reduced mobility imposed by the immobilization. A strategy to overcome this limitation and, at the same time, increment their catalytic activity, is the surface modification of the enzyme with small molecules like imidazolium or alkylated ammonium salts. However limited number of works have been reported in literature[11].

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Jia and co-workers performed the chemical binding of ionic liquids to the surface of lipase from *Porcine pancreas*[12]. They observed that the lipase modification bearing kosmotropic cations and chaotropic anions increased the enzymes' thermostability and enantioselectivity against the hydrolysis of racemic 1-phenethyl acetate.

The same strategy was applied to lipase B from *Candida antarctica* which revealed, after modification, a higher catalytic efficiency for the hydrolysis of *p*-nitrophenyl palmitate in aqueous medium. The thermostability and tolerance in organic media was also significantly improved by chemical modification of the enzymes' surface[13].

We have previously reported the positive effect of lipase TL PEGylation, on the lid destabilisation which allowed an easier access of the substrates to the active site, when synthesizing polyesters. The PEGylated form allowed to obtain a higher amount of polymer with a higher degree of polymerization in comparison with its native form[14].

Herein we aim to broaden the substrate chain-length specificity that lipase from *Thermomyces lanuginosus* can efficiently catalyse. Given that this lipase is used, in nature and in industry, for the hydrolysis of long-chain substrates, improving its catalytic performance for other substrates, would increment its range of applications in diverse fields. The degradation of fats in detergency or the chiral organic synthesis[15] are some of the applications that would benefit from this improvement. Thus, we chemically modify, for the first time, the surface of lipase TL by attaching linkers with differentiated chain length and hydrophobicity (naphthyl isothiocyanate, naphthaldehyde, butyraldehyde, hexyl aldehyde and dodecyl aldehyde) to the surface lysines and evaluated its activity and stability after modification.

A range of long-chain differentiated substrates were used for the evaluation of the modified enzyme's activity, following by the calculation of the kinetic parameters. Molecular dynamics simulations were performed to evaluate the impact of the modifications on the global conformation of the enzyme. Docking experiments were used to calculate the binding energies between the substrates and the native and modified lipases.

Experimental

Materials and methods

Lipase from *Thermomyces lanuginosus* (solution, ≥ 100,000 U/g), dodecyl aldehyde, naphthyl isothiocyanate, octyl isothiocyanate, phenethyl isothiocyanate, sodium cyanoborohydride, *p*-nitrophenyl butyrate, *p*-nitrophenyl laurate, *p*-nitrophenyl palmitate, *p*-nitrophenol and 2,4,6-trinitrobenzene sulfonic acid (5 % (w/v) in H_2O) were purchased from Merck. Naphthaldehyde, butyraldehyde, hexyl aldehyde, benzoyl chloride, *p*-nitrophenyl acetate and *p*-nitrophenyl hexanoate were obtained from TCI Chemicals and *p*-nitrophenyl octanoate from Alfa Aesar. All compounds were obtained with high purity and used without further purification. Prior to use, the native lipase is ultrafiltrate for the removal of any additives present. Ultrafiltration was performed with ultracel 10 kDa ultrafiltration discs composed of regenerated cellulose, 47 mm (Millipore) with ultrapure water (Milli-Q).

Chemical modification of lipase from *Thermomyces lanuginosus*

With isothiocyanates: The enzyme (2 mL) was added to 5 mL of sodium carbonate buffer (pH 9, 0.1 M), followed by the addition of the isothiocyanate (10 μL, or 5 mg in 50 μL of DMSO). The solution was kept at 4 °C overnight with vigorous stirring. The precipitates were then removed by filtration, followed by ultrafiltration (10 kDa). The modified enzymes are recovered after 48 h of freeze-drying.

With aldehydes: The enzyme (2 mL) was added to 5 mL of phosphate/$NaBH_3CN$ buffer (pH 5.1, 0.1 M sodium phosphate, 0.02 M $NaBH_3CN$), followed by addition of the aldehyde (4:1 w/w aldehyde: lipase). The solution was kept at 4 °C overnight with vigorous stirring[16]. The precipitates were removed by filtration, followed by ultrafiltration (10 kDa). The modified enzymes are recovered after 48 h of freeze-drying.

Synthesis of *p*-nitrophenyl benzoate: To a solution containing *p*-nitrophenol (100 mg, 0.72 mmol) in dry pyridine (2 mL) was added the benzoyl chloride (108 μL, 0.94 mmol) in an ice bath. The solution remained with stirring at 0 °C for 1 h, under nitrogen atmosphere, followed by rt overnight. The reaction was then stopped with the addition of water, and then the pyridine was removed using a rotary evaporator (Heidolph, Germany). Chloroform is then added, and the solution washed with $NaHCO_3$ (5x). The organic layer is dried over $MgSO_4$, filtered and the solvent removed in the rotary evaporator, affording the product as a white solid (quantitative yield). ^{1}H NMR (DMSO-d_6) δ_H: 7.62 (t, *J*= 7.6 Hz, 2H), 7.62 (d, *J*= 8.8, 2H), 7.77 (tt, *J*= 7.2, 1.2 Hz, 1H), 8.15 (dd, *J*= 8.4, 1.2 Hz, 2H), 8.35 (d, *J*= 9.2 Hz, 2H) ppm.

Modification efficiency

The modification of the lipases was evaluated by reaction with 2,4,6-trinitrobenzene sulfonic acid (TNBSA), following the procedure reported by Castillo et al[17].

MALDI-TOF

MALDI-TOF mass spectra were acquired on a Bruker Autoflex Speed instrument (Bruker Daltonics GmbH), as reported by Noro et al[18]. Sinapic acid was used as matrix, and the samples analysed in the linear positive mode, between 0-35 kDa m/z range.

Protein modification and SDS-PAGE

The quantification of the protein concentration and SDS-PAGE electrophoresis of all lipases were performed as described previously by Noro et al[14].

Enzyme activity

Small length substrates (*p*-nitrophenyl acetate to *p*-nitrophenyl octanoate): The activity was measured at 37 °C, in a final volume of 4 mL, containing the desired substrate (*p*-NPAcetate, *p*-NPButyrate, *p*-NPHexanoate or *p*-NPOctanoate at 6 mM), the enzyme (1 mg of protein) and the assay buffer (K_2HPO_4 buffer, pH 7.8, 50 mM). The reaction was initiated by the addition of the enzyme and stopped with the addition of acetone. The hydrolysis of the substrate was monitored by the formation of the *p*-nitrophenol at 400 nm. The measurements were conducted in a Synergy Mx Multi-Mode Reader from BioTek (USA) in a 96 well plates. One unit of enzyme activity is defined as the amount of enzyme which catalyses the production of 1 µmol *p*-nitrophenol from the initial substrate per minute. All substrates are clear solutions at the concentration used.

Large length substrates (*p*-nitrophenyl laurate and *p*-nitrophenyl palmitate): For large length substrates, the activity was measured in the same conditions that previously detailed but using the following assay buffer: K_2HPO_4 50 mM, pH 7.8, containing 0.5 mM of deoxycholic acid and 0.05 % w/v of gum arabic. After the reaction was stopped with acetone, the suspensions were filtered, followed by measuring the *p*-nitrophenol formed at 400 nm. All substrates are clear solutions at the concentration used.

Kinetic parameters

The enzymes' activity was measured following the procedure described previously. A range between 1 to 350 mM of *p*-NPAcetate, *p*-NPButyrate and *p*-NPOctanoate as substrates were used. The enzyme concentration was kept constant in all assays (1 mg/mL), performed at 37 °C. The maximum rate (V_{max}), the Michaelis-Menten constant (K_M), the turnover number (k_{cat}) and the catalytic efficiency (η) were determined after plotting the Michaelis-Menten curve. All calculations were obtained resourcing to GraphPad Prism 5.0 software (La Jolla, CA, USA), with at least 3 independent experiments performed.

Molecular Dynamics simulations

Molecular Dynamics (MD) simulations were performed on lipase from *Thermomyces lanuginosus* (lipase TL, PDB ID: 1TIB)[19] and on its modified form, with a dodecyl chain in position 98 (TL5), to understand the role of this linker on the lid and active site cavity. Previous work indicated that the Lysine in position 98 directly interferes with the activity pocket and lid configuration[14]. TL5 structure were optimized and GROMOS parameters generated, using ATB server (Automated Force Field Topology Builder)[20, 21]. Modified TL was designed using PyMOL[22]. The lipase with the linker (TL5) was then simulated in the simple point charge (SPC) water model, at 310 K, during 60 ns. The simulations options and algorithms are described in our previous work[14], as well as the simulation performed for the native TL. All simulations were performed using the GROMACS 5.1.4 version[23, 24], within the GROMOS 54a7 force field (FF)[25, 26]. From MD simulations, we computed a cluster analysis from GROMACS package, with the single-linkage method, to determine the middle structure of each lipase (TL and TL5). These representative structures were used for docking experiment. Electrostatic potential surfaces were calculated using the PDB2PQR server[27] and images generated in PyMOL, with the APBS plugin[28].

Molecular docking and complexes simulations

The substrates used for the hydrolytic activity, *p*-nitrophenyl butyrate and *p*-nitrophenyl octanoate were optimized using Gaussian09[29] software, through a DFT calculation with B3LYP/6-31+G(d,p) basis set[30]. The optimized structures were transferred to pdbqt format (atomic coordinates, partial charges and AutoDock atom types) for the use on docking protocol. Docking experiments were performed with AutoDock 4.0[31, 32] and prepared with the AutoDock Tools Software[31, 33]. The middle structures from TL and TL5 MD simulations were used as macromolecules and the substrates used as ligands. Lamarckian Genetic Algorithm (LGA) was chosen as search algorithm[34]. A grid box was created and centered at the activity site, in a resolution of 0.375 Å, with the necessary size to involve all catalytic triad and the different substrates. Grid potential maps were calculated using AutoGrid 4.0. Each docking consisted of 100 independent runs, with a maximum number of $25x10^5$ energy evaluations and a maximum number of 27,000 generations. Complexes simulations were performed using the best docking pose for each case, *i.e.* when the substrate is more internalized in the cavity and interacting with the catalytic triad. To simulate the complex, we also generated GROMOS parameters for the substrates using ATB. The complexes were minimized and equilibrated using the same options above described. MD simulations were carried out during 10 ns for each enzyme-substrate complex; the sufficient to perceive the spontaneous tendency of the substrate to remain or leave the active site. The interactions and distances between the substrates and the catalytic triad were followed.

Linkers simulations

The linkers used to obtain TL1 to TL5 were designed, optimized, and simulated in water, to understand which characteristics could have a role on enzyme activity, by changing the lipase and the environment. These 5 linkers were designed without the Lys core (see Table 1) and optimized using DFT calculations and Gaussian 09[29]. After this procedure, the linkers were submitted to ATB to generate GROMOS topologies and MD simulations were carried out during 10 ns, using the protocol previously described. From MD simulations trajectories, we computed the solvent accessible surface area (SASA), to determine the percentage of hydrophobic area of the linkers used to modify TL1 to TL5. This percentage correspond to the hydrophobic area divided by the total area of each linker, to enable a direct comparison between all cases. This information combined with experimental results reveals a tendency on activity accordingly to the linker profile.

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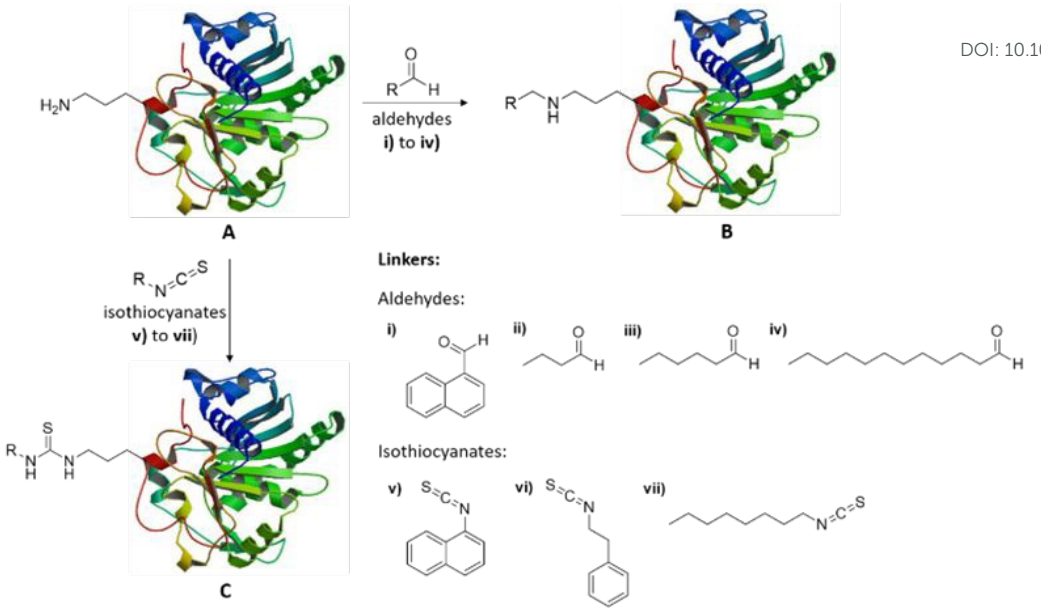


Figure 1. Proposed reactional scheme for the enzyme modification: **A)** native enzyme with an exposed lysine residue represented; **B)** enzyme modified with an aldehyde in the lysine residue, leading to a secondary amine; **C)** enzyme modified with an isothiocyanate in the lysine residue, leading to a thiourea; linkers used for lipase modification: **i)** naphthaldehyde; **ii)** butyraldehyde; **iii)** hexyl aldehyde; **iv)** dodecyl aldehyde; **v)** naphthyl isothiocyanate; **vi)** phenethyl isothiocyanate; **vii)** octyl isothiocyanate.

Half-life time ($T_{1/2}$)

The $t_{1/2}$ of each enzyme was performed by incubating the lipase solution in a water bath at 60 °C (1 mg/mL, pH 7.8). The activity was then calculated as previously described[35]. The stability of the enzymes in solution at 4 °C and 37 °C through the months was also evaluated. Both assays were evaluated by measuring the hydrolytic activity of *p*-nitrophenyl butyrate at 37 °C, as mentioned before.

Effect of the pH on the activity

The enzymes were incubated in the respective pH (range of pH 1.81 to 10.5) under universal buffer (Britton-Robinson buffer[36]) at 37 °C. After incubation for 10 min at the respective pH, the activity of the enzyme was measured at 37 °C in phosphate buffer (pH 7.8) as mentioned above, using *p*-nitrophenyl butyrate, *p*-nitrophenyl octanoate or *p*-nitrophenyl palmitate as substrates.

Effect of the temperature on the activity

The enzymes were incubated in the respective temperature (range of 10 °C to 70 °C) in a Thermal Shaker like (VWR) with phosphate buffer (pH 7.8). After incubation for 10 min at the desire temperature, the activity of the enzyme was measured at 37 °C using *p*-nitrophenyl butyrate, *p*-nitrophenyl octanoate or *p*-nitrophenyl palmitate as substrates.

Circular dichroism

The native and modified lipases from *Thermomyces lanuginosus* were studied by circular dichroism (CD) spectroscopy, using a Jasco J-1500 spectropolarimeter. The sample preparation and parameters used were followed as reported by Noro et al[14].

Fluorescence measurements

The intrinsic fluorescence of all lipases was conducted by their excitation at 280 nm. The emission spectra were set between 300 and 600 nm. All enzymes were analysed with a final concentration of 0.2 μM, dissolved in phosphate buffer (K_2HPO_4 buffer, pH 7.8, 50 mM).

Results and discussion

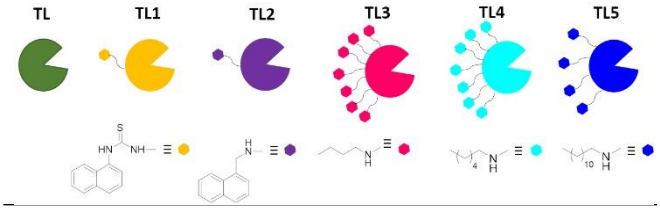
The covalent modification of enzymes modulates their function and properties, being the catalytic performance greatly influenced by the properties of the modifiers. The nature, size and hydrophobicity of the linkers is expected to alter the enzyme performance at different levels depending also on the degree of modification.

Herein lipase from *Thermomyces lanuginosus* (TL) was firstly reacted with compounds (linkers) from two different classes: isothiocyanates and aldehydes, with different chain length and hydrophobicity. The linkers were chosen based on their high reactivity and ability to covalently bond to the primary amines of the exposed lysine residues of the enzyme (total of 7 residues observed by molecular dynamics). The proposed scheme for the reactions between the enzyme and the tested linkers is represented in Figure 1.

The degree of modification was assessed by the TNBSA assay and by MALDI-TOF spectrometry (Table 1 and Figure S1).

After product isolation and evaluation (MALDI-TOF and TNBSA) the data revealed that all aldehyde compounds tested were successfully linked to the enzyme, with modification of at least one lysine available (Table 1).

Table 1. Modification degree and number of modified residues evaluated by MALDI-TOF and TNBSA assay, being TL the native enzyme, and the modified enzymes: TL1 modified with naphthyl isothiocyanate, TL2 modified with naphthyl aldehyde, TL3 modified with butyraldehyde, TL4 modified with hexyl aldehyde and TL5 modified with dodecyl aldehyde



	MALDI-TOF (values in Da)	Nº of residues modified by MALDI-TOF	% of modification (by TNBSA assay)	Nº of residues modified by TNBSA
TL	29620	-	-	-
TL1	29771	1	8	1
TL2	29826	1	15	1
TL3	30283	7	93	7
TL4	30392	7	97	7
TL5	30270	4	49	3-4

The aliphatic isothiocyanates (Figure 1, **vi** and **vii**) demonstrated the lowest reactivity, confirmed by the isolation of non-modified lipase. One unit of a more reactive aromatic isothiocyanate (Figure 1, **v**) was grafted onto lipase enzyme's surface. We can assume, as it is postulated, that isothiocyanate compounds, when linked to the amine group of the lysine residue, give rise to thioureas, whereas aldehydes give rise to secondary amines[14, 37]. Lysine residues are the most nucleophilic amines present in enzymes[38]. The chosen class of compounds (aldehydes and isothiocyanates) are described to be preferentially linked to this residue instead to other reactive amino acids[38].

Different reactivities between linkers might explain the differentiated modification degrees obtained experimentally.

MALDI-TOF results revealed, in comparison with the non-modified lipase (29620 Da), an increase of the molecular weight of the enzyme after modification with all the compounds used. In all cases, the results of MALDI-TOF and TNBSA are in accordance, revealing a direct relation between the degree of modification with the number of residues modified (Table 1).

The lipases modified with a naphthyl moiety (TL1 and TL2) revealed only one modified residue, probably due to their poor solubility and/or low reactivity of the starting material.

TL3 and TL4 (with butyl and hexyl chains, respectively) were modified in all the 7 available lysines, while TL5 (with dodecyl chains) was only modified in 4 of the 7 available lysines.

The length of the size chains may induce a steric effect, which might explain the different modification degree obtained for the tested linkers. Lysines located at positions surrounded by larger amino acids, are less prone of being modified by larger linkers. One may predict that, in the case of aldehydes, the degree of enzyme modification is inversely proportional to the size of the linker chain.

The modified lipases were also analysed by SDS-PAGE to evaluate size and purity (Figure S2). All enzymes were obtained pure, revealing only one visible band at around 30 kDa. The small molecular weight of the linkers is not detectable by SDS-PAGE and thus no significant differences between modified lipases were observed.

Hydrolytic activity

Absolute activity

The hydrolytic activity of a fixed amount of lipase (1 mg) was evaluated against seven substrates, differing in the size of the aliphatic chain (from 0 to 16 carbons) (Figure 2A). The results are presented in U/mg after incubation at 37 °C for 1 min (Figure 2B and 2C).

From the results depicted in Figure 2, it is possible to perceive that the modification of lipase with the different linkers allowed to improve the hydrolytic activity of the enzyme against substrates with differentiated chain-length, which are not normally hydrolysed by the native lipase. One may observe that for all the enzymes tested the highest hydrolytic activity was achieved against the medium-length chain substrates, *p*-nitrophenyl hexanoate (*p*-NPH, 6C) and *p*-nitrophenyl octanoate (*p*-NPO, 8C). Activity values higher than 100 U/mg were reached for all the lipases.

An exception is observed for the enzyme TL5. This enzyme, modified with four dodecyl chains, revealed higher activity against all the substrates tested, comparing with the native lipase. For the small chain-length substrates, *p*-nitrophenyl acetate (*p*-NPAc, 2C) and *p*-nitrophenyl butyrate (*p*-NPB, 4C), the hydrolytic activity obtained was at least 3-fold higher than obtained with the native enzyme. For these two substrates, all other modified enzymes showed activity values similar to the native enzyme. It is also worth mentioning that this modified lipase (TL5) also displayed activity (13 U/mg) against a synthetic substrate (*p*-nitrophenyl benzoate, 0C).

For higher chain-length substrates (*p*-NPL and *p*-NPP), from all the tested enzymes, TL5 displayed also the highest hydrolytic activity (2-fold).

For *p*-NPL, TL2, TL3 and TL4 revealed higher activity than native lipase (2-fold of activity increase). These three enzymes displayed activity values of ≥100 U/mg, for substrates containing 6, 8 and 12 carbons in the aliphatic chain, while the native enzyme only reached this activity for *p*-NPH (6C) and *p*-NPO (8C).

Lipase from *Thermomyces lanuginosus* is an enzyme composed by a lid over its active site, requiring interfacial activation. Moreover, the active site is surrounded by hydrophobic residues, which hinder its activity against small hydrophilic substrates. These features make lipase TL more able to hydrolyse longer substrates[5].

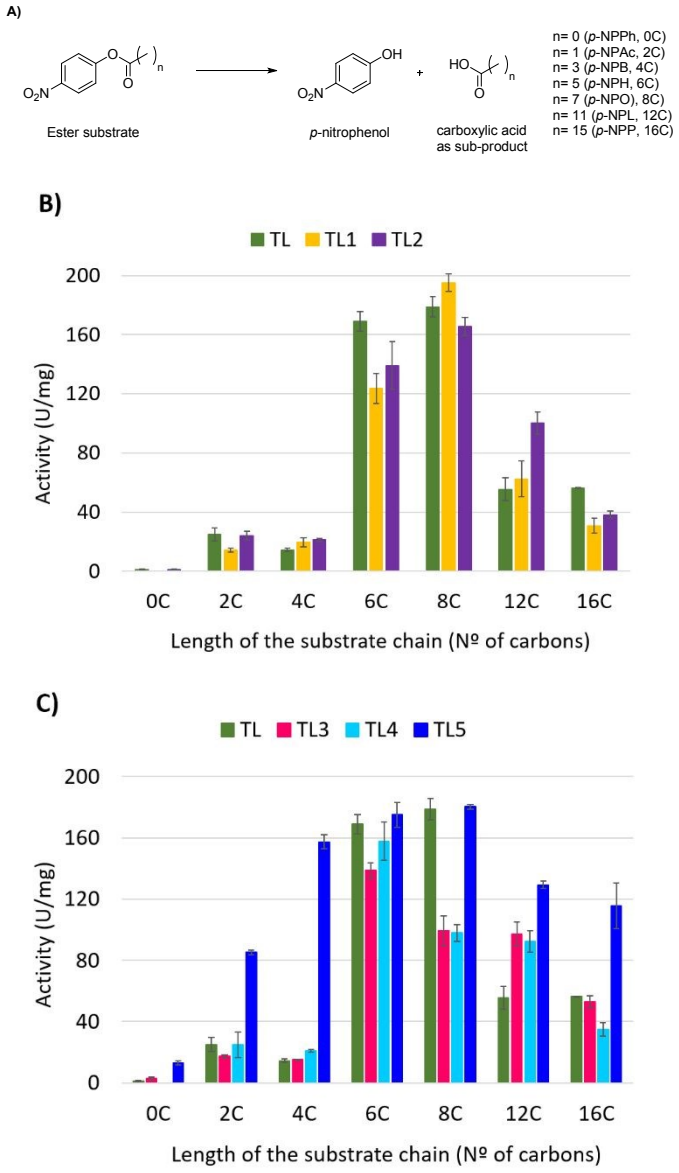


Figure 2. A) Proposed reactional scheme representing the hydrolysis of the ester substrates and the designation of each substrate according to the number of carbons in the aliphatic chain; **B)** Absolute hydrolytic activity of native lipase (TL) and of modified lipases with aromatic linkers (TL1 and TL2), and **C)** with aliphatic linkers (TL3-TL5), measured against different substrates: *p*-nitrophenyl benzoate (*p*-NPPh, 0C), *p*-nitrophenyl acetate (*p*-NPAc, 2C), *p*-nitrophenyl butyrate (*p*-NPB, 4C), *p*-nitrophenyl hexanoate (*p*-NPH, 6C), *p*-nitrophenyl octanoate (*p*-NPO, 8C), *p*-nitrophenyl laurate (*p*-NPL, 12C) and *p*-nitrophenyl palmitate (*p*-NPP, 16C).

This lipase has a lysine residue near the lid (residue number 98 of the enzymes' sequence), which is a plausible position for modification with the tested linkers. Given the linkers' hydrophobicity, possible Van der Waals interactions with the lid and/or the hydrophobic surface surrounding the active site can occur, leading to different activity performances depending on the linker used. In this way, molecular dynamics tools were applied herein to infer the hydrophobicity of the linkers and correlate this parameter with the hydrolytic activity displayed by the modified lipases.

Figure 3 depicts the hydrophobic area of the linker versus the hydrolytic activity of the modified lipases against three different substrates, representative of short, medium and long chain-length: *p*-nitrophenyl acetate (*p*-NPAc), *p*-nitrophenyl hexanoate (*p*-NPH) and *p*-nitrophenyl laurate (*p*-NPL).

From the data obtained it can be perceived that the hydrolytic activity of the enzyme modified with a specific linker is directly related with the percentage of its hydrophobic area. The highest overall activity was obtained for the lipase TL5 modified with the highly hydrophobic linker, 4 dodecyl chains (hydrophobic area higher than 70 %). We may assume that this linker can interact more favourably with the lid and/or with the hydrophobic residues surrounding the active site, eliminating the need for interfacial activation. These findings might explain the excellent catalytic performance of TL5 for all substrates, especially for the short-chain substrates. Together with an easier access of the small substrates, a slight increase of the active site size may also occur, so that longer substrates can be better accommodate and further hydrolysed.

All other linkers tested are less hydrophobic than the dodecyl chain differing in their hydrophobic area in about 10 % (between 55-65 %). TL3 (butyl chains) and TL4 (hexyl chains) are completely modified, which indicates that the lysine near the lid is alkylated. However, these linkers are less hydrophobic than the dodecyl chain (TL5) which can consequentially lead to a weaker interaction with the hydrophobic environment around the active site. We may infer that these modified enzymes still required interfacial activation, given that vestigial activities were achieved when used against the shorter chain substrates. The same assumption might be valid for TL1 and TL2 (both with a naphthyl moiety), in which only one lysine residue was modified.

Comparing with native lipase the most pronounced activity differences regarding TL2, TL3 and TL4, were observed for *p*-NPL. For this substrate, the three enzymes showed activity values 2-fold higher than native enzyme. It is predicted that the modification of these lipases induced a slight change in the size cavity of the active site, which might explain the highest activity measured. Nevertheless, the effect of the cavity size is still less pronounced than the observed for TL5 when tested against longer substrates (*p*-NPP, 16C).

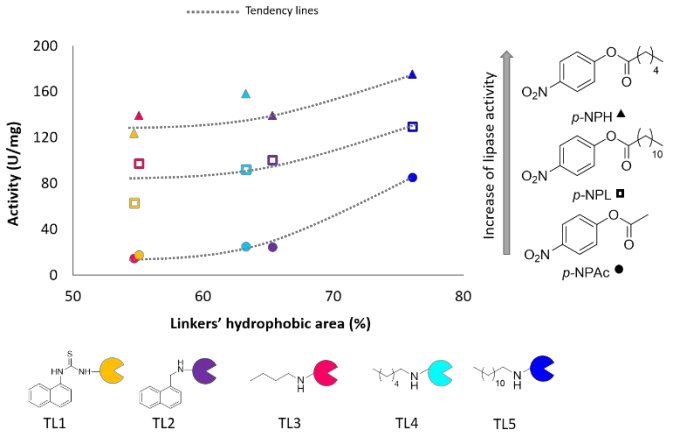


Figure 3. Activity of the modified lipases versus hydrophobic area of the linkers; activity measured against *p*-nitrophenyl acetate (*p*-NPAc, •), *p*-nitrophenyl hexanoate (*p*-NPH, ▲) and *p*-nitrophenyl laurate (*p*-NPL, ◘). The colour of the symbols in the graphs corresponds to the colour of each modified enzyme represented bellow the graph.

TL1 did not displayed superior activity that the native enzyme in neither of the tested substrates. This modified lipase is only mono substituted with an isothiocyanate group. This modification might occur in a lysine placed away from the active site enabling any destabilization of the lid nor of the active site cavity.

It is important to state that the presence of the free aldehyde or isothiocyanate in the medium did not induced any alteration on the enzymes' activity, confirming that the performance differences observed are induced by the surface modifications undertaken. Previously we have PEGylated lipase TL, through modification of the surface lysines with a monofunctional aldehyde-PEG (5000 Da). The modified lipase displayed a higher polymerase activity for the synthesis of a polyester, poly(ethylene glutarate), comparing to the native form. However, the hydrolytic activity of the modified lipase against *p*-nitrophenyl butyrate, remained similar to the native enzyme[14]. The surrounding PEG allowed an easier access of the monomers to the active site, nevertheless, the substrate used for hydrolytic activity evaluation revealed similar accommodation in the active center for both native and PEGylated form. Contrarily to PEG, which is an hydrophilic macromolecule, the linkers herein studied are small hydrophobic compounds which, depending on the size, may influence the enzyme conformation and therefore its hydrolytic activity.

As postulated by some authors, a proportional increment of the enzymes' activity with the degree of modification would be expected[12]. However, for the lipase studied, this behaviour was not observed, since higher levels of modification did not correspond to the best performance results (comparing TL5 with TL3 and TL4). One may assume that the activity is not only dependent on the level of modification but is extremely influenced by the linker type, chain size, hydrophobicity, and positioning at the enzyme's spatial.

Kinetic parameters

The kinetic parameters of the lipase modified with the different linkers were evaluated against different substrates: *p*-nitrophenyl acetate, *p*-nitrophenyl butyrate and *p*-nitrophenyl octanoate (Table 2). The substrates were chosen based on the preliminary activity results obtained for the modified lipase, where we observed an increase in the enzymes' activity over the grown of the substrate chain length.

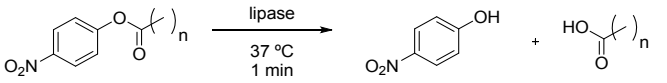
Given the data obtained from Table 2, and as expected, the best kinetic parameters were obtained for *p*-NPO. For this substrate, the highest V_{max} is achieved with TL1, however, with a low K_M and catalytic turnover, revealing that this enzyme displayed the lowest catalytic performance for *p*-NPO comparing to all other enzymes tested. The K_M values of TL2, TL3, TL4 and TL5 are similar to the values of the native enzyme, being the catalytic turnover similar or lower after modification.

For the shorter substrates, TL5 displayed the best catalytic performance. The lowest K_M value (23 mM) is obtained for *p*-NPB, being 5-times lower than the K_M value obtained for the native enzyme (110 mM). Moreover, the highest catalytic turnover (Figure 4) is observed for this modified enzyme against *p*-NPB (η= 23364 M^{-1} s^{-1}), which is 9 times higher than the value obtained for the native enzyme (η= 2552 M^{-1} s^{-1}). All other modified enzymes showed a catalytic turnover similar to the native lipase.

For *p*-NPAc, the K_M value of TL5 (128 mM) is half of the K_M value of the native enzyme (245 mM). This modified enzyme revealed a catalytic turnover 6-fold higher than TL, whereas no significant alterations of the kinetic values were verified for the other modified enzymes.

As previously stated, lipase from *Thermomyces lanuginosus* is usually used for hydrolytic purposes in its immobilized form. The immobilization strategy increases its specific activity comparing to its free form, however some kinetic parameters are negatively affected[39, 40]. The strategy here applied does not restrict the enzymes' conformation and, in some cases, even improves its kinetic parameters (TL5).

Table 2. Kinetic parameters of native and modified lipases (V_{max} (μmol/mg/min), K_M (mM) and η= k_{cat}/K_M (M^{-1} s^{-1})) calculated for the hydrolysis of *p*-nitrophenyl acetate (*p*-NPAc), *p*-nitrophenyl butyrate (*p*-NPB) and *p*-nitrophenyl octanoate (*p*-NPO) under the conditions: substrate concentration varied between 1 and 350 mM, enzyme content (1 mg), performed at 37 °C for 1 min



n=1 (*p*-NPAc, **2C**)
n=3 (*p*-NPB, **4C**)
n=7 (*p*-NPO, **8C**)

p-nitrophenol

carboxylic acid as sub-product

***p*-nitrophenyl acetate (*p*-NPAc)**			
Enzyme	V_{max} (μmol/mg/min)	K_M (mM)	η (M^{-1} s^{-1})
TL	752	245	1517
TL1	610	214	1412
TL2	680	223	1516
TL3	780	299	1316
TL4	503	253	1005
TL5	2535	128	9828
***p*-nitrophenyl butyrate (*p*-NPB)**			
Enzyme	V_{max} (μmol/mg/min)	K_M (mM)	η (M^{-1} s^{-1})
TL	566	110	2552
TL1	626	129	2402
TL2	335	63	2656
TL3	554	126	2218
TL4	708	229	1563
TL5	1061	23	23364
***p*-nitrophenyl octanoate (*p*-NPO)**			
Enzyme	V_{max} (μmol/mg/min)	K_M (mM)	η (M^{-1} s^{-1})
TL	1426	56	12482
TL1	1671	125	6612
TL2	1022	45	11340
TL3	701	42	8439
TL4	610	34	9018
TL5	1391	53	12979

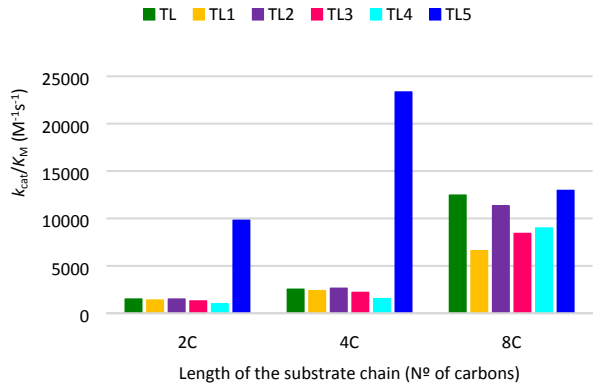


Figure 4. Catalytic turnover (η= k_{cat}/K_M) of the native and modified enzymes calculated for the hydrolysis of *p*-nitrophenyl acetate (*p*-NPAc, 2C), *p*-nitrophenyl butyrate (*p*-NPB, 4C) and *p*-nitrophenyl octanoate (*p*-NPO, 8C).

The kinetic profile of lipase TL is described to be improved as the chain-length of the substrates grows[41], as we also confirmed. Moreover, the modified enzymes obtained revealed better kinetic performance than the native enzyme, mainly for the shorter substrates.

The kinetic parameters of the modified lipases are, as mentioned previously, greatly influenced by the type of linker attached and its positioning at the enzymes' surface. The activity data also indicated that the lack of hydrophobicity of the small substrates seems to be counterbalanced by the hydrophobic character of the linker, as the highest activity differences were observed for smaller and less hydrophobic substrates. For longer and hydrophobic substrates, the impact of the enzyme modification (TL5) seems to be irrelevant since the access to the enzymes' active site is always ensured (Figure 4).

In order to predict the enzyme performance and better understand the conformational changes induced by the modifications, molecular dynamics simulations were performed on TL and TL5.

Molecular dynamics simulations

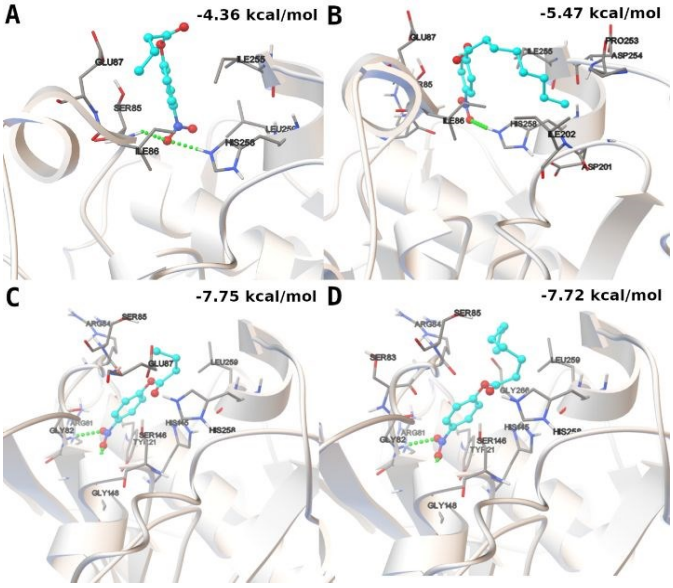
MD simulations were carried out using a similar strategy applied on previous work using the same enzyme[14]. During simulations, no modifications were carried out for native TL (PDB ID: 1TIB), whereas for TL5, the enzyme was modified at Lys98 with a dodecyl chain (Figure S3A). This specific lysine is in one of the lid arms and any modification may cause important alterations of the lid arrangement or of the size/opening of the active site, which is crucial for the enzymes' activity.

Figure S3 highlights the lid, the catalytic triad, and the linker in the case of TL5 (Figure S3C). In these middle structures we observe that the helical lid undergoes some unfolding on its structure in both enzymes, nevertheless leading to a larger cavity for TL5 (yellow filling), in which the amino acids side chain positioning also contributes to a well-defined cavity. This conformational change was followed through RMSD and RMSF analysis (Figure S4), where a RMSF peak is seen around the residue 98 in TL5. Globally, the two lipases are stable enzymes in aqueous medium. The larger cavity obtained for TL5 may be responsible for the increase of the activity for longer substrates (*p*-NPL and *p*-NPP), since a better accommodation of their long aliphatic chain can occur. The same can be stipulated for TL2, TL3 and TL4, that are more active than the native TL for *p*-NPL. However, the increase of the cavity size of TL2, TL3 and TL4 is not so pronounced than for TL5, explaining the lower activity of these enzymes for the longer substrate tested (*p*-NPP).

Noteworthy is that the change in the cavity size and conformation of TL5 might induced the great increase of its activity against small substrates (*p*-NPAc and *p*-NPB), resulting from an easier access of these substrates and a higher affinity to the active site.

Using PyMOL were found two possible accesses to the active site: one from the top and another from the left lateral side, under the lid. To monitor the possibilities of binding and the dynamics, the molecular docking of the substrates to the lipases was performed, followed by the simulation of the resulting complexes.

Docking experiments were carried using two substrates: *p*-NPB (4 carbons) and *p*-NPO (8 carbons). The obtained results agree with the experimental data, revealing a higher affinity of both substrates with the active site of TL5 (- 7.7 kcal/mol), where a more stronger interaction takes place (Figure 5, C-D), while the native enzyme presented lower binding energies (< 3 kcal/mol). Furthermore, native TL also showed lower values of energy for *p*-NPB (-4.36 kcal/mol) than for *p*-NPO (-5.47 kcal/mol), as expected, given the activity values observed experimentally (14.4 U/mg and 175 U/mg, respectively).



During simulations, one can observe that *p*-NPB is more unstable in TL, leaving rapidly from the active pocket, remaining stable in TL5. The *p*-NPO substrate remains stable in the pocket in both cases.

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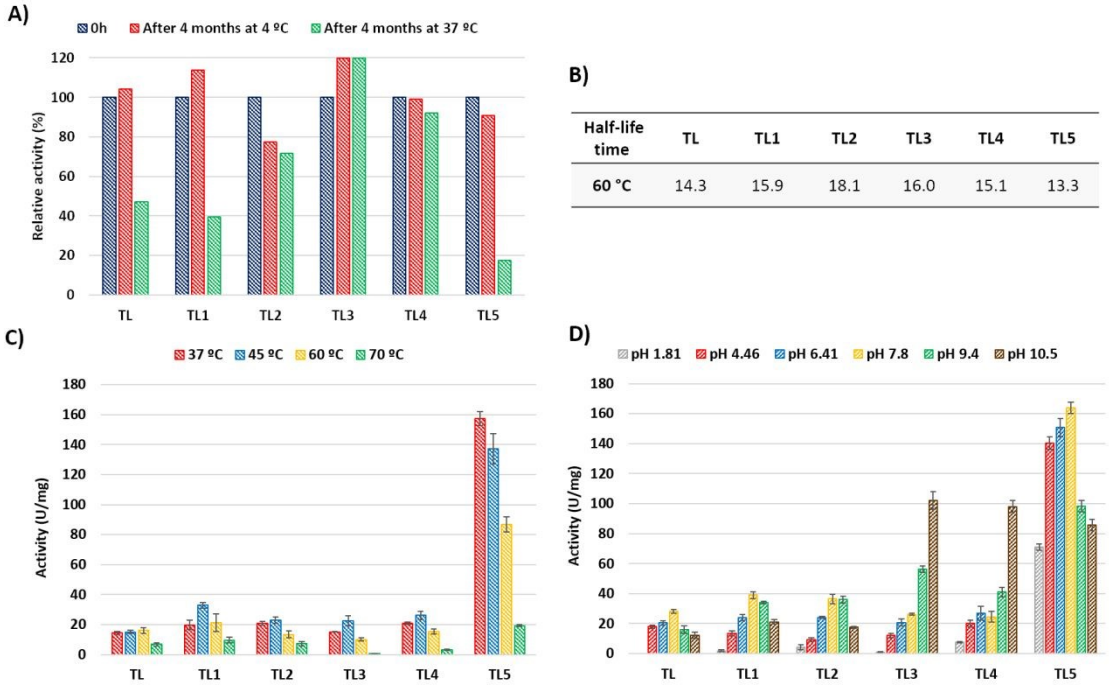
Figure 5. Representation of the best docking poses, interactions and $\Delta G_{binding}$ between *p*-nitrophenyl butyrate with native TL (**A**) and with TL5 (**C**); *p*-nitrophenyl octanoate with native TL (**B**) and with TL5 (**D**). Enzymes are represented in grey cartoon, sticks to highlight the amino acids residues participating in the binding, substrate in cyan ball and sticks, and hydrogen bonds in green dashed.



Half-life time	TL	TL1	TL2	TL3	TL4	TL5
60 °C	14.3	15.9	18.1	16.0	15.1	13.3

Figure 6. Stability of native and modified enzymes: **A)** Relative activity (%) at time zero, and after 4 months in solution at 4 °C and 37 °C; **B)** Half-life time (in hours) of the enzymes at 60 °C; **C)** Effect of temperature in the enzymes' activity after 10 min incubated at the respective temperatures (37 °C, 45 °C, 60 °C and 70 °C); **D**) Effect of pH in the enzymes' activity after 10 min incubated at the respective pH (1.81, 4.46, 6.41, 7.80, 9.40 and 10.5). The results presented were obtained using *p*-nitrophenyl butyrate as substrate.

The distance between the substrates and the Ser146 residue of to the catalytic triad, was measured along time (Figure S5). The results confirm what is observed visually following the simulation trajectories in PyMOL, *i.e*. the spontaneous unbinding of *p*-NPB in TL (purple line), indicating a weaker interaction than with TL5 (blue line). For *p*-NPO, similar results were obtained.

Stability of TL and modified lipases

The stability over time of TL and modified lipases was firstly evaluated at different temperatures: 4 °C, 37 °C and 60 °C. All enzymes revealed remarkable stability in solution at 4 °C, with minimum activity lost after 4 months (Figure 6A). At 37 °C, after 4 months, at least 20 % of their initial activity endures. Despite a similar hydrolytic activity and kinetic profile, TL2 showed a higher thermostability compared with native enzyme. After 4 months at 37 °C, the activity of this enzyme is still almost 2-fold higher than the native enzyme. All enzymes modified with an aromatic or aliphatic aldehyde revealed higher thermostability than the native lipase.

At 60 °C, the activity lost is more pronounced, allowing to calculate the half-life time of the enzymes (Figure 6B). With exception of TL5, all lipases revealed higher half-life time than native TL. Despite the lower half-life time obtained for TL5, the specific activity of this modified form is still higher than native (30 U/mg *vs* 6 U/mg).

Lipase from *Thermomyces lanuginosus* it is known by its broad range of optimum pHs, from 7 to 10, with an optimum temperature at around 40 °C.

The effect of temperature (10-70 °C) and pH (1.8-10.5) in the lipases' activity were investigated (Figure 6C and 6D) both using *p*-nitrophenyl butyrate as substrate. From the data obtained it is possible to perceive a similar activity trend for all the enzymes tested.

Regarding temperature, all enzymes showed similar activity against *p*-nitrophenyl butyrate between 37 °C and 45 °C, presenting only residual activity at 70 °C. These results agree

with the thermal stability associated with lipase TL. A similar trend is observed when other differentiated chain-length substrates were used, such as *p*-nitrophenyl octanoate (8C, *p*-NPO) and *p*-nitrophenyl palmitate (16C, *p*-NPP) (Figure S6A and S6B, respectively).

Regarding pH, TL is completely inactive while all modified enzymes showed some residual activity against *p*-nitrophenyl butyrate at the lowest pH tested (pH 1.81). At this pH, TL1, TL2, TL3 and TL4 showed activity values bellow 6 U/mg, while TL5 maintains remarkable activity (70 U/mg). These findings agree with the literature reports, which describe that the chemical modification of enzymes improve their stability. A similar tendency among native and modified lipases (TL1-TL4) is observed when a larger substrate (*p*-NPP) was used at the lowest pH. The activity of TL5 is still 4-fold higher than TL (Figure S6D).

All enzymes revealed similar activity values against *p*-NPO at pH 1.81.

The most surprising result is the activity obtained for TL3 (butyl linkers) and TL4 (hexyl linkers) at the highest pH tested (9.4 and 10.5). The hydrolytic activity of these lipases increases 4-fold comparing to the activity obtained at standard pH (7.8). Both enzymes suffered a complete modification of their lysines, and the buffer pH change will drastically affect their total charge, inducing therefore alterations on the activity. Circular dichroism was applied to study this behaviour. From the results obtained one may observe that the increase of pH does not led to any significant change in the enzymes' conformation (data not shown), which keep a profile similar to native lipase. Moreover, lipase from *Thermomyces lanuginosus* is known for its broad range of activity until pH 10, indicating that no denaturation should occur at higher pHs. Regarding this, the increase in the hydrolytic activity of TL3 and TL4 at higher pH values, may be caused by the different charge acquired upon modification.

The pKa value of the lysine residue is reported to be around 10.4[42]. Also, the pKa of the lysines are highly influenced by many factors, such as position, surrounded amino acids, etc[43]. After alkylation, pKa values are expected to be different, and therefore, different charges are estimated.

In order to confirm these allegations, we used molecular dynamics simulations (Figure S7) to evaluate the effect of the enzymes' total charge on their hydrolytic activity, by calculating the pKa by Poisson-Boltzmann electrostatics.

This tool allowed to infer the positioning of the most significant charge variations on the lipases structure. From Figure S7, we may observe that when the lysines are alkylated, the most affected area is the surrounding active site. TL3 and TL4 showed an increase in their activity at higher pH for all the substrates tested (4C, 8C and 16C). These results may indicate that at higher pH, the alkylation of the lysines together with an enlargement of the active site and/or an easier access of the substrates might occur, and thus the interfacial activation may no longer be necessary. We may assume that the similar phenomenon previously postulated for TL5 may occur for TL3 and TL4, however, with greater influence of the different charge acquisition induced by pH fluctuations. It is well known that the secondary structure of the proteins is ensured by the hydrogen bonds between amino acids. Amine residues are crucial in this phenomenon[44]. It is possible that small structural changes might occur near the active site due to changes in the residues' charge, improving the access of the substrate to the active site, and therefore enhancing the enzymes' activity.

Circular dichroism

Circular dichroism (CD) was performed in order to evaluate the effect of the modifications performed on the conformational structure of the enzymes (Figure S8). The CD spectra of the native enzyme showed the expected behaviour, described in literature[45]. Lipase TL is structurally composed by a central eight-stranded, mostly by parallel beta-sheets and five interconnecting alpha helices[5]. All modified enzymes (TL1 to TL4) revealed a profile similar to the native TL, with no significant modifications in the number of α-helices and β-sheets. Given the experimental results obtained for these enzymes, these results were expectable.

TL5 revealed the most discrepant profile, with the lowest intensity obtained. The decrease in the intensity can be related to a slight unfolding of the protein's structure. Considering the experimental activity results, one may infer that the access of the active site, is facilitated by the modification with the dodecyl chain at the enzymes' lid, which enhances its activity for short and long substrates. This behaviour was also observed by molecular dynamics simulation that confirmed the active site enlargement and a consequent easy access of the substrates.

Fluorescence analysis

The intrinsic fluorescence of the lipases, related to the presence of fluorophore residues, was considered, as a strategy to observe conformational fluctuations[46] induced by the compounds grafted to the lysine residues (Figure S9). Comparatively to native lipase, all the modified lipases showed a decrease of the fluorescence intensity, which is usually considered to be associated to an unfolding of the protein. However, the fluorescence intensity can be also affected by other factors, such as morphology, protein size and protein aggregation[47]. By circular dichroism it was only possible to observe a slight unfolding of TL5, indicating that the decrease of fluorescence for all other modified lipases might be related to other factors.

A hypochromic blue shift (from 320 to 316 nm) was observed for TL5, while TL3 and TL4 showed their maximum intensity at the same wavelength than the native enzyme, or at a higher wavelength (TL1 and TL2). The blue shift is frequently associated to the exposure of the amino acids to a more hydrophobic microenvironment, in which the fluorophore residues are more internalized[48]. The tryptophan residue at the lid of lipase TL is one of the amino-acids responsible for the enzymes' fluorescence[49]. Changes in the microenvironment of this residue may influence the fluorescence, which given the differences obtained in terms of activity, might indicate that the lid of the enzyme was modified. Since TL5 is modified with the most hydrophobic linker, its fluorescence decreases greatly induced by a more hydrophobic microenvironment.

Conclusions

In this work, chemical modifications of lipase from *Thermomyces lanuginosus* with different linkers (isothiocyanates and aldehydes) were for the first time explored to improve activity, stability, and affinity to differentiated substrates. The results showed that aldehyde linkers were more prone to modify the lysines of lipase comparing with the isothiocyanates. One concluded that the size and hydrophobic character of the linkers influenced greatly the enzyme activity. The longer and hydrophobic is the linker, the stronger is its interaction with the hydrophobic amino acid residues near the active site, conducting to a destabilization of the lid and to an enlargement of the active site's cavity. This is expected to induce an improvement of the lipase performance.

The implementation of the easy methodology developed, broadening the substrates chain-length specificity, increments thereafter the range of substrate possibilities that can be hydrolysed by this lipase, paving the way to the establishment of new industrial applications.

Conflicts of interest

There are no conflicts to declare.

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