A EUROPEAN JOURNAL OF CHEMICAL BIOLOGY

CHEMBIOCHEM

SYNTHETIC BIOLOGY & BIO-NANOTECHNOLOGY

Accepted Article

Title: Enlightening the role of subdomains in the catalytic behavior of lipases/acyltransferases homologous to CpLIP2 through rational design of chimeric enzymes

Authors: Anne-Hélène Jan, Eric Dubreucq, and Maeva Subileau

To be cited as: *ChemBioChem* 10.1002/cbic.201600672

Link to VoR: http://dx.doi.org/10.1002/cbic.201600672

A Journal of

ChemPubSoc Europe

WILEY-VCH

www.chembiochem.org

Enlightening the role of subdomains in the catalytic behavior of lipases/acyltransferases homologous to CpLIP2 through rational design of chimeric enzymes

Anne-Hélène Jan,[a] Éric Dubreucq,[a] and Maeva Subileau*[a]

Abstract: The lipases/acyltransferases homologous to CpLIP2 of *Candida parapsilosis* catalyze efficiently acyltransfer reactions in lipid/water media with high water activity (a_w > 0.9). The characterization of two new enzymes of this family, CduLAc from *Candida dubliniensis* and CalLAc8 from *Candida albicans,* was performed. Despite their 82% identity, these 2 enzymes have significant differences in their catalytic behavior. To understand the role played by the different subdomains of these proteins (the main core, the cap and the C-term flap), chimeric enzymes were designed with rational exchanges of cap and C-term flap, between CduLAc and CalLAc8. Results showed that the cap region played a significant role in the substrate specificity and the main core was found to be the most important part of the protein in the acyltransfer ability. Similar exchanges were made with CAL-A from *Moesziomyces (Candida) antarctica* but only the C-term exchange was successful. Yet, the role of the C-term was not clearly evidenced, except that it is essential for the activity.

Introduction

The development of sustainable processes for the production of fuels and chemicals has become a challenge for research and industry. This is particularly true in oleochemistry where traditional technologies require abundant organic solvent usage and harsh reaction conditions [1]. To overcome these customary routes, the utilization of lipases offers interesting alternative [2]. Lipases (E.C. 3.1.1.3) naturally catalyze the hydrolysis of triacylglycerols (TAG) into glycerol and free fatty acids (FFA) and can be used for synthesis reactions, mostly in conditions where hydrolysis is limited (in organic solvent). Lipases can exhibit wide substrate spectrum, good chemo-, regio- and stereoselectivity and eventually high stability in organic media [3]. These extensive properties make them valuable biocatalyst for industrial applications.

Lipase engineering first aimed at improving their properties toward tough reaction conditions mimicking the traditional oleochemistry processes [1]. More recently, the possibility to use peculiar lipases that preferentially catalyze transesterification reactions in water as solvent and in mild-conditions has appeared very promising to reach higher reaction yields and rates in greener conditions [4]. Within this aim our research team has evidenced a group of lipases/acyltransferases homologous to CpLIP2 from *Candida parapsilosis* [4a, 5]. CpLIP2 was the first lipase/ acyltransferase described [6], on the basis of which we have produced and characterized several mutants and natural variants [4a, 7]. Recently we have described a simple methodology to assess the acyltransferase potential of lipases that allowed consistent sorting of the tested enzymes in three classes, with high, medium and low acyltransferase ability [4a]. Lipases/acyltransferases display medium to high acyltransferase activity and are phylogenetically related to the *Candida albicans* lipase-like family of the lipase engineering database (LED) [8] with more than 55 % of identity in their primary sequences (lipases with high sequence similarity are assigned to a single homologous family in the LED). They also share low, but significant sequence similarity with the LED superfamily of *Moesziomyces (Candida) antarctica* lipase A (CAL-A) [8], although CAL-A was shown to display only low acyltransferase ability [4a]. Apart from their exceptional property to catalyze the transesterification reaction at higher rate than hydrolysis in aqueous media (a_w > 0.9), the lipases/acyltransferases display various substrate specificities: medium or long chain selectivity for the acyl-donor substrate, a preference for unbranched primary alcohols as acyl-acceptor [9]. In addition, CpLIP2 was also shown to be able to catalyze the acyltransfer reaction to other nucleophiles than alcohols, such as peptide [10] or hydroxylamine [11]. Like other standard lipases, lipases/acyltransferases could thus be implemented in a wide range of applications.

Lipases of the CAL-A LED superfamily[8] exhibit no significant sequence similarity with other LED superfamilies and are predicted to share with CAL-A its structural peculiarities[7a, 12]: CAL-A is a globular class Y α/β serine hydrolase of 436 amino-acids, and possesses, in addition to the main core of the protein, two distinct sub-domains called the “cap” and the “flap” [12-13]. In comparison to other α/β hydrolases, CAL-A and its homologs have very divergent sequences, particularly in the cap region which is exceptionally long (about 100 residues) [12].

The CAL-A cap was described to comprise a hydrophobic tunnel-like binding site for the acyl moiety, comparable to that of the *Candida rugosa* lipases (CRL) [12], and mutations in the cap domain indeed allowed modification in the substrate specificity profiles[4b]. The CAL-A flap is composed of 2 β-strands in the C-terminal region and was also compared to the lid in CRL [12]. This flap was recently proposed to play a “lid-like” role in the interfacial activation of the enzyme [13b].

To better understand the role and importance of these 2 structural subdomains in substrate and reaction specificities of the lipases and lipases/acyltransferases of the CAL-A LED

[a] Dr. A.-H. Jan, Prof. E. Dubreucq, Dr. M. Subileau
Montpellier Supagro, UMR 1208 IATE
2 place Viala, 34060 Montpellier cedex 2, France
E-mail: maeva.subileau@supagro.fr

Supporting information for this article is given via a link at the end of the document.

superfamily [8], we decided to perform "subdomains exchanges" by rational design of chimeric genes. To generate these enzymes, the main challenge was to find biocatalysts presenting differences in their catalytic behaviour but also sharing enough identity in their primary sequences to allow the production of functional chimeric enzymes. Searching in the lipases/acyltransferases family, we have identified two new enzymes meeting these requirements: CduLAc from *C. dubliniensis* and CalLAc8 from *C. albicans*. A similar strategy has already been successfully conducted in lipases from *C. rugosa* (CRL) [14], *Bacillus subtilis* (lipase A) [15], and *M. (C.) antarctica* (lipase B) [16]. Compared to the wild enzymes, when functional, these chimeric biocatalysts exhibited changes in specific activity and substrate specificity, confirming the important role of the lid in the stability and the selectivity of the enzymes [15]. In these studies the swapped sequence comprised less than 30 amino-acids. Here we describe functional exchanges of about 10 to 100 residues sequences that allowed the modulation of the stability and substrate specificity of the enzymes. Our results also give interesting insights into the putative respective functions of the cap and flap in this original group of lipases.

Results and Discussion

Choosing the candidate enzymes for rational exchanges of sub-domains: CduLAc and CalLAc8

Recently, 2 new lipases/acyltransferases were identified by our group: CduLAc from *Candida dubliniensis* [7b] and CalLAc8 from *Candida albicans* [4a]. These lipases share 82 % identity in their primary sequence and 56 % and 58 % with CpLIP2, for CalLAc8 and CduLAc respectively. Like CpLIP2, CalLAc8 and CduLAc 3D models have a structure similar to that of CAL-A, with a α/β hydrolase fold that holds a catalytic triad (serine, histidine, aspartic acid), a cap and a flap subdomains.
The high identity in their sequences and structures conjugated with differences of reactivity and specificity that will be presented in the following characterization, make of these two biocatalysts models of great interest for the elucidation of structural determinants involved in some of the lipases/acyltransferases peculiarities, and to generate the chimeric enzymes with rational sub-domains exchanges.

Design of chimeric enzymes to assess the role of the subdomains in the lipases/acyltransferases

Referring to the structure of CAL-A (2VEOA.PDB) and CpLIP2 3D model [7a], sequence alignment and 3D modelling suggest that, within the proteins of the LED superfamily[8], 3 subdomains can be identified: the main core, the cap and the C-term flap [7a, 12-13]. The cap comprises about 100 residues organized in α helices (6 in CAL-A) and the flap domain consists of a 10 to 25 amino-acids C-terminal sequence. Within the variants of the CAL-A LED superfamily, the most variable regions are those of these putative caps and flaps (Table S1). Besides, as said earlier, the CAL-A flap was compared to the lid of *C. rugosa*

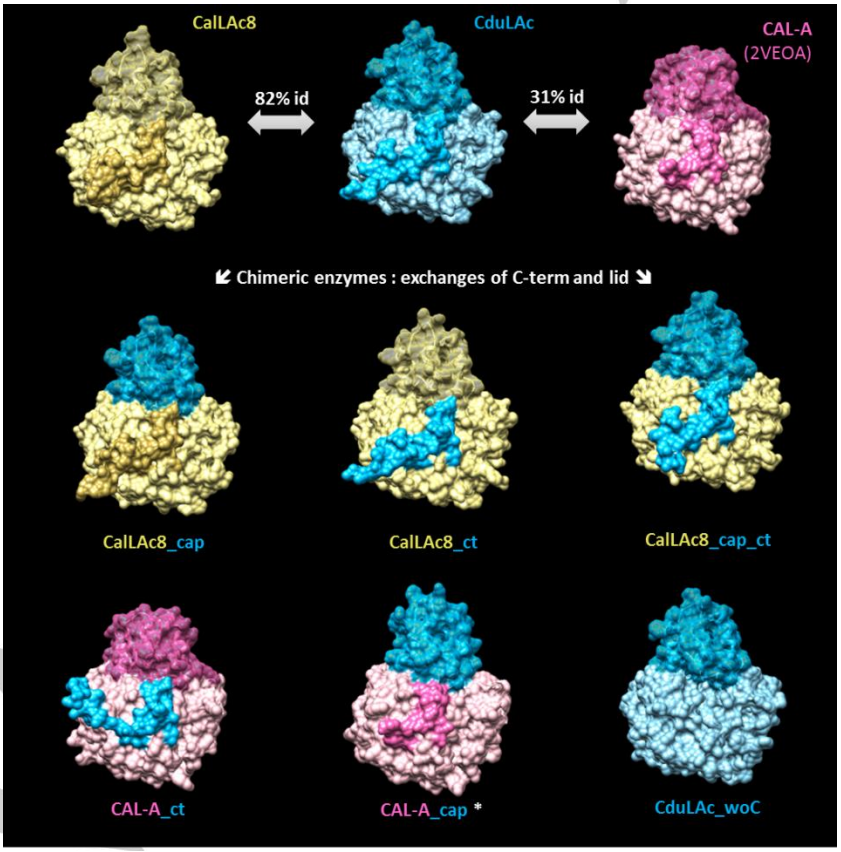


Figure 1 Design of chimeric enzymes based on the wild-type CalLAc8, CduLAc and CAL-A.
* no lipase activity detected in the screening conditions

lipases, which covers the active site and prevents direct access to the substrate binding site[12].

In the CAL-A LED superfamily, the C-term flaps are variable in composition and length (Table S1, Figure S1). In the lipases/acyltransferases (CpLIP2, CtroL4, CalLAc8, CduLAc and CalLAc5) the C-term is 10 to 15 residues longer than in the classical lipases such as CAL-A or AflaL0[9] and is not structurally well defined. It is somewhat reminiscent of the mobile lid-like α-helix of the CAL-B family that displays low similarity within the family, and only few contacts with the rest of the structure[16]. We hypothesized that the main core, cap and flap of the studied enzymes could play different roles in the substrate and reaction specificities.

To investigate how each sub-domain is involved in the catalytic behavior of the lipases/acyltransferases, the chosen strategy was to use CduLAc and CalLAc8 as models, because they are very similar in sequence but exhibit significant differences in their acyltransfer ability and chain-length selectivity. Based on their sequences and 3D models, we created chimeric enzymes with rational exchanges of sub-domains (caps and flaps) (figure 1). CAL-A was added to the study for the creation of additional chimeric enzymes (figure 1). CAL-A served as reference to evaluate the transferability of the chosen strategy to enzymes more variable in sequence. It also appeared particularly interesting because the catalytic behavior of CAL-A is significantly different from those of the lipases/ acyltransferases: its acyltransfer ability is low, its substrate specificity is centered on myristic acid (C14:0), and it exhibits exceptional thermostability. The overall strategy could allow the production of new biocatalysts with interesting specificities. Comparison of the catalytic characteristics of the wild and chimeric enzymes could facilitate the elucidation of structure/function relationships within the subdomains and the identification of hot spots that could be targeted to rationally modify the catalytic specificities.

More precisely, regarding the C-term flap, its beginning was determined based on sequences alignments of the proteins of the family (Figure S1). We selected the most variable region that corresponded to the last 29 amino acids in CduLAc. To observe the influence of the C-term flap and its length, we designed a mutant of CduLAc "without C-term", lacking the 29 amino acids of the C terminal end (CduLAc_woC). We also substituted the C-term of CalLAc8 and CAL-A with the one of CduLAc (CalLAc8_ct and CAL-A_ct, respectively).

The cap was defined based on the description of the structure of CAL-A by Widmann et al [12]. To study the role played by the cap in the substrate specificity, we designed two chimers: the cap of CduLAc was grafted on the main cores of CalLAc8 and CAL-A (CalLAc8_cap and CAL-A_cap, respectively). Unfortunately, no lipase activity was detected for CAL-A_cap, perhaps because these two subdomains were not compatible, the caps of CAL-A and CduLAc being too different (only 12% id). At last, to evaluate the conjugated effect of both C-term flap and cap, we built a mutant with the main core of CalLAc8 and the cap and C-term flap of CduLAc (CalLAc8_cap_ct). All the mutants were subcloned in *Komagataella (Pichia) pastoris* X33 and functional enzymes were produced and characterized, except for CAL-A_cap.

Characterisation of the wild and chimeric enzymes to assess structure/function relationships of subdomains.

Transesterification activity in aqueous medium

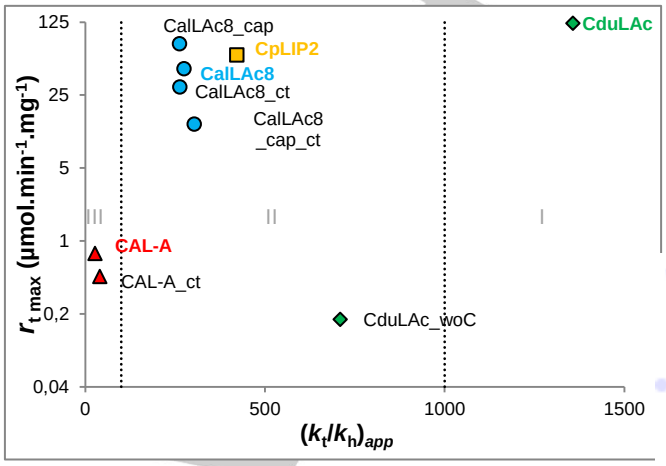


Figure 2. Efficiency of the enzymes: $r_{t\,max}$ of the lipases (log scale) in function of $(k_t/k_h)_{app}$. I, II and III areas correspond to high, medium and low acyltransferase ability. Detailed results are given in table 1. The most efficient biocatalysts for acyltransfer activity shall exhibit high $r_{t\,maxt}$ and high $(k_t/k_h)_{app}$.

First, we evaluated the influence of the change of subdomains on the acyltransfer ability of the enzymes (figure 2, table 1). The acyltransfer activity of CalLAc8 and the chimeric enzymes was assessed precisely with ethyl oleate in aqueous emulsion in the presence of various methanol concentrations between 0 and 4.5 M at 30°C, and compared with those of CduLAc and CAL-A previously published [7b, 9]. Each enzyme was characterized by (i) the transfer ratio (TR) at the various methanol concentrations tested, that corresponds to the relative initial rate of alcoholysis compared to the sum of initial rates of alcoholysis plus competitive hydrolysis, (ii) the alcohol concentration required to reach 50 % of TR ($[MeOH]_{TR50}$) and (iii) the ratio of the apparent rates constant of acyltransfer versus hydrolysis $(k_t/k_h)_{app}$ [4a, 17]. Figure 2 represents the efficiency of the enzymes to catalyze acyltransfer. The higher the $r_{t\,max}$ (maximum specific transfer rate) and $(k_t/k_h)_{app}$ are, the more efficient the lipase is. As shown on figure 2, despite 82 % identity in their primary sequences, CalLAc8 is less efficient to catalyze acyltransfer than CduLAc with ethyl oleate as a substrate. Indeed, its $[MeOH]_{TR50}$ is 5 times higher and its $r_{t\,max}$ 3 times lower than those of CduLAc. CalLAc8 belongs to the class II lipases/ acyltransferases [4a], that are characterized by medium acyltransferase ability with $(k_t/k_h)_{app}$ between 100 and 1000 and $[MeOH]_{TR50}$ between 0.05 and 0.5 M, like the lipase/acyltransferase of reference CpLIP2. In comparison, CduLAc belongs to class I (high acyltransferase ability), exhibiting $(k_t/k_h)_{app}$ superior to 1000 and $[MeOH]_{TR50}$ inferior to 0.05 M. Moreover, as methanol concentration increased up to 1 M, the specific activity of CduLAc was enhanced, while for CalLAc8 the maximal activity was reached with no methanol (figure S2) and decreased when methanol was added.

Table 1. Results of the experimental determination of kinetic constants and acyltransfer abilities of the enzymes: (i) the ratios of the apparent rate constants of acyltransfer versus hydrolysis $(k_t/k_h)_{app}$ and $(k_t/k_h)_{app'}$ are given (respectively related the nucleophiles concentrations and thermodynamic activities), as well as (ii) methanol concentrations and (iii) corresponding thermodynamic activities of methanol (a_{MeOH}) and water (a_W) at TR_{50} and (iv) maximum specific transfer rate ($r_{t\,max}$). Based on the $(k_t/k_h)_{app}$ and $[MeOH]_{TR50}$, the enzymes are graded in 3 categories of transfer ability: class I lipases/acyltransferases with $[MeOH]_{TR50}$ inferior to 0.05 M (in green), class II lipases/acyltransferases with $[MeOH]_{TR50}$ between 0.05 and 0.5M (in blue), class III lipases with $[MeOH]_{TR50}$ superior to 0.5M (in red). Reactions were conducted in phosphate buffer (pH 6.5, a_W> 0.9) at 30°C, in the presence of 10 µmol.mL^{-1} ethyl oleate and 0.01-4.5 M methanol.

Enzyme	$r_{t\,max}$ (µmol.min^{-1}.mg^{-1})	$(k_t/k_h)_{app}$	$(k_t/k_h)_{app'}$	at TR50 [MeOH] (M)	at TR50 a_{MeOH}	at TR50 a_W	Class
CduLAc	121	**1357**	607	0.04	0.002	0.999	**I**
CduLAc_woC	0.2	**710**	315	0.08	0.003	0.999	**II**
CpLIP2	60.4	**422**	189	0.13	0.005	0.998	**II**
CalLAc8_cap	77	**263**	136	0.19	0.008	0.997	**II**
CalLAc8	45	**275**	123	0.20	0.008	0.996	**II**
CalLAc8_cap_ct	13	**303**	117	0.20	0.008	0.996	**II**
CalLAc8_ct	30	**263**	118	0.22	0.009	0.996	**II**
CAL-A_ct	0.5	**40**	20	1.20	0.047	0.978	**III**
CAL-A	0.8	**27**	13	1.92	0.074	0.966	**III**

The 18% differences in the two enzymes primary sequences are therefore responsible for significant differences in their acyltransfer ability and their level of activity in the presence of methanol (increase or decrease).

Regarding the role of the C-term flap, the $r_{t\,max}$ of CduLAc_woC was 615 times lower than that of the wild enzyme, meaning that the presence of the C-term is crucial for the enzymatic activity of CduLAc and probably for the overall protein structuration. Moreover, its $(k_t/k_h)_{app}$ was 2 times lower than the one of CduLAc showing a significant decrease of the acyltransfer ability. When comparing the 3D models of CduLAc and CduLAc_woC, we observed that the nucleophile pocket previously identified in the main core of the protein[7c] appears obstructed by the C-term flap in the wild enzyme. By removing the 29 last amino acids, in addition to a general destabilisation, we might have created an easier and larger entrance to the active site, facilitating its accessibility for the lipid substrate (and the water), through the nucleophile pocket. The decrease of specific activity might be linked to the steric hindrance of the lipid substrate inside the nucleophile pocket, limiting the accessibility for the acyl acceptor (docking experiments, not shown). About the swapping of the C-term flaps, the mutant CalLAc8_ct had comparable catalytic behaviour to its wild-type enzyme CalLAc8 (figure 2, table 1). In this case, the length of the C-term was the same as the wild type and the modification of amino acids composition did not have much impact. In comparison, we observed that the mutant CAL-A_ct was characterised by $r_{t\,max}$ in the same range as that of CAL-A, but that the $(k_t/k_h)_{app}$ of this chimeric biocatalyst was 1.5 times higher than that of the wild-type enzyme. It thus appeared that, in the case of CAL-A_ct the change in composition and length of the C-term flap (14 additional residues compared to CAL-A) had a significant impact on the acyltransfer ability. Therefore it appears that the C-term somewhat plays a role in the acyltransfer ability and that the overall size of this subdomain might be more important than the amino-acid composition. In addition, although the exchanges of C-term modified numerous amino-acids, its effect was nothing comparable to those previously described with only punctual modifications of the amino acids comprised in the nucleophile pocket [7c].

To evaluate the influence of the cap on the acyltransfer activity, we studied the mutant CalLAc8_cap. While the value of $(k_t/k_h)_{app}$ was similar for CalLAc8 and CalLAc8_cap, the $r_{t\,max}$ was 2 times higher. Looking at the specific activities as a function of the methanol concentration, we observed that the increase of activities was proportional for hydrolysis and transesterification, with comparable profile (Figure S2). The difference of composition between CalLAc8 and CalLAc8_cap might thus not affect the nucleophile attack (and the acyltransfer ability) but the formation of the acyl-enzyme (and the total specific activity), suggesting that the lipid substrate binding site is in the cap.

Conjugated effects of the cap and the C-term were observed with CalLAc8_cap_ct. Its $r_{t\,max}$ value was 3 times lower and $(k_t/k_h)_{app}$ similar to the one of CalLAc8. The mutations of both cap and C-term flap might have destabilized the structure of the enzyme but no significant effect on the acyltransfer behavior could be evidenced.

It is to be noted that none of the CalLAc8 chimeric enzymes were activated in the presence of methanol, unlike what was observed with CduLAc[7b]. This activation phenomenon could thus not be transferred with these sub-domains exchanges.

Therefore, it seems that the cap does not play a significant role in the acyltransfer ability of the enzymes while the effect of the C-term flap remains unclear. Our results tend to show that it is the main core of the protein that probably has the greatest influence on the specificity of reaction and also on the activation/inhibition phenomenon. This observation is to be related to the nucleophile pocket recently described by Jan et al [7c]. Indeed, although very similar, the nucleophile pockets of CduLAc and CalLAc8 differ by 18% of their residues, and notably by the key residue A366 of CduLAc [7b], which is a threonine in CalLAc8.

Effect on the nature of the accepting alcohol on transesterification

As the ability of the enzymes to catalyse acyltransfer can also depends on the nature of the nucleophile, their behavior in the presence of various alcohols was evaluated. The competition between water and alcohol as acyl acceptor is modulated by both the chemical reactivity and the thermodynamic activity of the reactants and by intrinsic properties of the biocatalysts that will preferentially catalyze either hydrolysis or alcoholysis reactions in the deacylation step of the catalytic mechanism.

Thus, to evaluate only the enzyme-substrate interactions and catalytic features, the tested alcohols were added in the reaction mixture at the same relative thermodynamic activities ($a_{alcohol}/(a_w + a_{alcohol})$). The relations between the alcohol concentrations and thermodynamic activities were estimated by the UNIFAC group contribution method in the biphasic medium, using liquid-liquid equilibria parameters (table 2). For a same thermodynamic activity, the alcohol concentration can be very different (*e.g.* 7 fold variation between ethanol and butanol).

Table 2. Relative thermodynamic activity of alcohols $a_{alcohol}/(a_{alcohol}+a_w)$ and the corresponding alcohols concentrations. Relation between thermodynamic activities asnd concentrations were estimated using UNIFAC- LLE taking into account the presence of alcohol, water and ester in a biphasic system at 30°C.

alcohol	relative thermodynamic activity of alcohol (%)	alcohol concentration (M)
Ethanol	1.0%	0.079
	2.9%	0.24
Propan-1-ol	1.0%	0.029
	2.9%	0.087
Propan-2-ol	1.0%	0,029
	2.9%	0,087
Butan-1-ol	1.0%	0,012
	2.9%	0,034
Butan-2-ol	1.0%	0,012
	2.9%	0,034
Isobutanol	1.0%	0,012
	2.9%	0,034

The transesterification of methyl oleate (C18:1 ME) in emulsion was performed in the presence of primary alcohols (ethanol, propan-1-ol, butan-1-ol and isobutanol) and secondary alcohols (propan-2-ol and butan-2-ol) at an estimated relative thermodynamic activity of alcohol of 1 % and 2.9 % (Table 2).

Figure 3 shows that the two wild enzymes, CalLAc8 and CduLAc displayed a transesterification activity toward all primary and secondary alcohols. For a similar relative thermodynamic activity of alcohol, the transesterification ratio increased with the length of the alkyl chain. The transesterification ratio also depended on the structural isomer tested. For both enzymes, TR was higher for primary than secondary alcohols. Comparing the effect of the increase of the relative thermodynamic activity of alcohol (1 % and 2.9 %), the hydrolysis activity decreased while the transesterification increased. Similar results were obtained with CpLIP2 by Neang et al [9]. As observed with the increase of methanol concentration in the first experiment of this study and in previous studies [7b], the total activities of CalLAc8 and CduLAc seemed to be sensitive to the increase of alcohol concentration. While the total activity of CalLAc8 decreased, the one of CduLAc was higher at 2.9 % than at 1 % of relative thermodynamic activity of alcohol. Apart from the differences evidenced in transesterification ratios and in the influence of the increase of alcohol concentration on the activity, CduLAc and CalLAc8 showed comparable preference for the acyl acceptor alcohol. For example, with the two enzymes the higher TR were always obtained with butan-1-ol and the lower with propan-2-ol. Therefore, although their structural differences play a significant role in the enzyme selectivity toward alcohol or water, resulting in medium or high acyltransferase character, they do not seem to influence peculiar selectivity for one alcohol or another.

The experiments conducted with various alcohols as nucleophiles did not show clear distinction between the enzymes, wild type and chimeric. Indeed, the specificity profiles of acyl acceptors were the same for all the enzymes (Figure S3). Previous works led on CpLIP2 evidenced that some amino acids of the cap, in contact with the main core of the protein and in the vicinity of the active site had an impact on the acyl acceptor specificity. For example, the mutation I231F in CpLIP2 caused a decrease of specificity for the secondary alcohols[7a]. Yet, as the

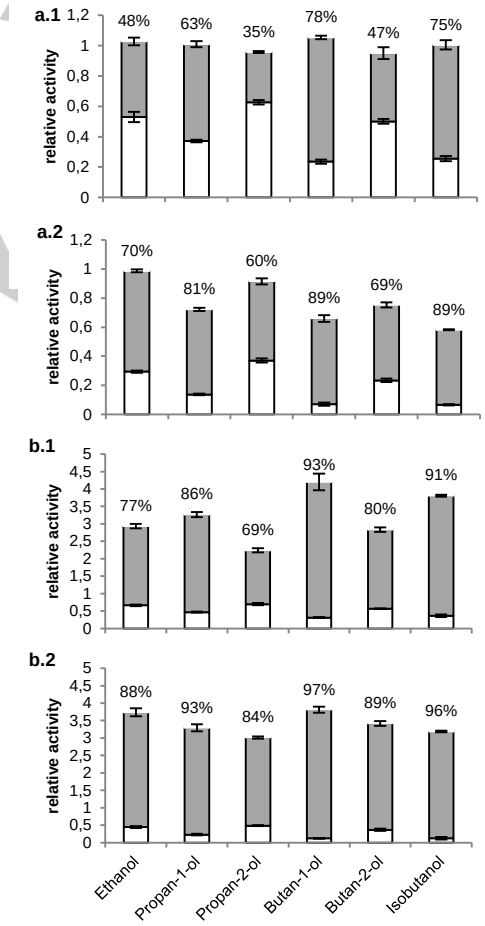


Figure 3 Transesterification (▫) and competitive hydrolysis (▫) activities catalyzed by (a) CalLAc8 and (b) CduLAc with various alcohols. Reactions were performed at 30°C, pH 6.5 in the presence of 10 mM of C18:1 ME and a relative thermodynamic activity of alcohol of 1 % (i.1) or 2.92 % (i.2). For each enzyme, values of transesterification and hydrolysis activity are expressed relatively to their specific activity in condition without alcohol (hydrolysis, not shown) and normalized to 1: (a) 15.96 ±0.23, (b) 11.8 ±0.44 $\mu mol.min^{-1}.mg^{-1}$

amino acids of the cap in contact with the main core of CalLAc8 are identical or at least share similar properties than the ones present in CduLAc, no difference in the alcohol specificity was observed in relation with this cap exchange. The only notable differences between wild-type and chimers were (i) the increase of TR for CAL-A_ct compared to CAL-A and (ii) the decrease of total activity for CduLAc_woC in comparison to CduLAc (Figure S3).

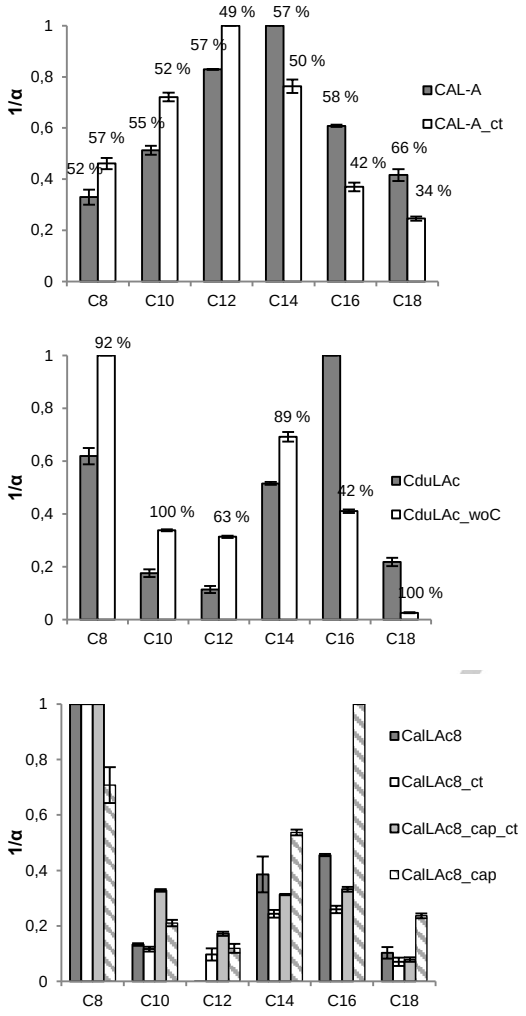


Figure 4 Relative specificity constants 1/α for total activity (transesterification plus competitive hydrolysis of ethyl esters of saturated fatty acids C8-C18 in presence of methanol) catalyzed by CAL-A, CduLAc, CalLAc8 and the chimeric enzymes (CAL-A_ct, CduLAc_woC, CalLAc8_cap, CalLAc8_ct and CalLAc8_cap_ct). Transesterification ratios are stated above the bars if not 100%. Reactions were performed during 15 min at 30°C pH 6.5 in the presence of 2.2 M of methanol and 10 mM of ethyl esters mixture (1.6 mM of each ester).

Influence of the acyl chain of the donor ester on the activity with saturated monoesters of various carbon chain length

The substrate specificity was assessed with an equimolar mixture of six saturated monoesters (from 8 to 18 carbon atoms) at pH 6.5 and 30°C in the presence of 2.2 M methanol. Figure 4 shows specificity constants 1/α [18] for total activity (transesterification plus competitive hydrolysis) catalyzed by the studied enzymes.

In these conditions, no residual hydrolysis activity was detected with CduLAc and CalLAc8, confirming the acyltransferase character of the two wild enzymes. Compared to the activity with ethyl oleate as a substrate, the activity on saturated monoesters was very low. Indeed, the total specific activities in the presence of 2.2 M methanol and ethyl oleate were of 61 $U.mg^{-1}$ and 20 $U.mg^{-1}$, 74 and 32 times higher than with saturated monoesters for CduLAc and CalLAc8 respectively. CduLAc specificity constants distribution was bimodal, with C16:0 EE as the best long-chain substrate and another optimum for C8:0 EE. CalLAc8 discriminated strongly between its preferred substrate, C8:0 EE, and the other monoesters, notably with a very low affinity for C12:0 EE (no activity detected in the tested conditions).

To bring insights into the understanding of these different substrate specificities, docking experiments were conducted with the 3D models of both wild lipases/acyltransferases. For each substrate, 150 possible docking positions were obtained as the result of 15 runs, each retaining the 10 best positions. The supposed mechanism of reaction is a nucleophilic attack by the oxygen atom of the hydroxyl group of the catalytic serine on the carbon atom of the carboxyl group of the ester. The docking was considered correct when the distance between these two atoms was inferior to 4 Å. In addition to the distance, the number of correct positioning for each molecule within the 150 results was considered to be an indicator of the probability of formation of the acyl-enzyme. It is to be noted that the docking calculations have to be considered cautiously, as they do not take into account the mobility of the molecules and represent only one putative arrangement of the residues. Yet, they can be used to support our experimental observations. In CduLAc, two tunnels inside the cap allowed the positioning of the substrates (Figure 5). The first tunnel (number 1 on the figure 5) is equivalent to the tunnel binding-site of CAL-A, in which was trapped a polyethylene glycol molecule in the CAL-A crystallized structure [13a] and that was compared to the CRL binding-site [12]. In this tunnel, C16:0 EE and C8:0 EE were in direct competition, with distances to the catalytic serine of 3.5 Å and 3.3 Å respectively. C12:0 EE was also docked in this tunnel at a distance of 3.5 Å. It has to be noted that the number of correct dockings obtained for the three substrates in the tunnel 1 can be correlated to the specificity constants. Indeed, more molecules of C8:0 EE and C16:0 EE were docked correctly, compared to C12:0 EE. In the second position inside the cap, referred as tunnel number 2 on figure 5, docking was only possible for C12:0 EE and C16:0 EE. No docking of C8:0 EE was correct in this tunnel. However, a third position (tunnel number 3 on figure 5) was identified in the cavity previously described as the nucleophile pocket [7c], in which only C8:0 EE was docked at a distance of 3.3 Å of the catalytic serine.

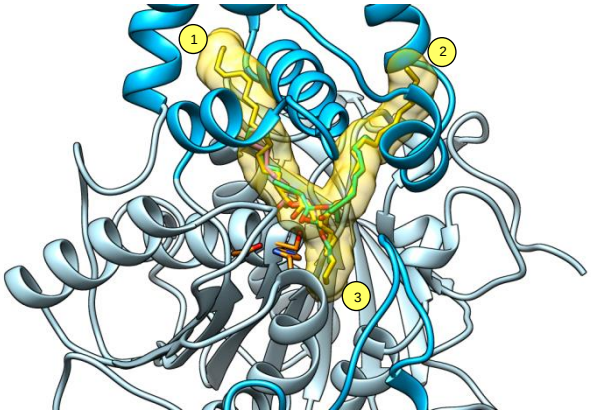


Figure 5. Docking of C8:0 (pink), C12:0 (green) and C16:0 (gold) in CduLAc. The catalytic triad is colored in orange and the cap and C-term flap in darker blue. The identified tunnels for the positioning of lipidic substrates are numbered 1 to 3 and highlighted in yellow.

On the contrary, only the first tunnel allowed the correct positioning of the substrates in CalLAc8 (data not shown). The dockings of C8:0 EE and C16:0 EE in tunnel 1 in CalLAc8 seemed to be similar to those in CduLAc but differed for C12:0 EE. Indeed while this substrate was successfully docked in CduLAc, no correct positioning for the reaction was obtained with CalLAc8. Moreover, the correct positioning of C8:0 EE was obtained with a higher probability than the one of C16:0 EE. Interestingly, the total specific activity of CalLAc8 is twice higher for C8:0 EE than C16:0 EE (0.31 U/mg and 0.15 U/mg), which matches the docking experiments. In comparison, the specific activities of CduLAc for the same substrates were 0.19 $U.mg^{-1}$ (C8:0 EE) and 0.28 $U.mg^{-1}$ (C16:0 EE). Regarding tunnel 2, it was only evidenced in CduLAc where it appeared to favor C16:0 EE. The substrate specificities of CduLAc and CalLAc8 observed experimentally were thus supported by the docking experiments in the 3D model developed. The cap appears to be the crucial sub-domain for the substrate specificity (lipid acyl-donor), as it was observed previously in CpLIP2, in CAL-A and in *C.rugosa* lipases [7a, 13a, 19].

Then, we evaluated the acyl donor specificity of the chimeric enzymes. The deletion of the C-term flap in CduLAc led again to a drastic decrease of total specific activity, from 0.82 $U.mg^{-1}$ to 0.04 $U.mg^{-1}$ respectively for CduLAc and CduLAc_woC. As observed on figure 4, the specificity constants for CduLAc_woC were higher for monoesters from C8:0 to C14:0. Based on the hypothesis that a new access to the active site for the lipid substrate is created, the absence of C-term flap might have increased the accessibility for short length substrates, so the competition between the esters is higher than in the wild type enzyme. Docking experiments conducted with C8:0 EE, C12:0 EE and C16:0 EE on CduLAc and CduLAc_woC showed that if the dockings in the cap were identical, there was possibly a larger access for the substrate to the active site. Indeed, in CduLAc_woC the absence of C-term shielding the active site suggest a possible binding of the lipidic substrate in a activity lying under the catalytic serine and previously described as the "nucleophile pocket" [7c] (data not shown). Concerning the mutant CAL-A_ct, the monomodal distribution was slightly modified, centered on C12 instead of C14 for the wild enzyme. Moreover, the 1/α of C8 to C12 were higher for CAL-A_ct than CAL-A and the 1/α of C14 to C18 decreased. With the chimeric CaLAc8_ct, no major change of specificity profile was observed. Indeed, only a minor decrease of 1/α for the non-preferred substrates (C10 to C18) was evidenced. The C-term of CduLAc seems therefore to be slightly more favorable to the short chain substrates. Indeed the increase of specificity for shorter substrates was related to an increase of specific activities for these substrates, while the ones for the longer substrates were more constant between the chimers and the wild-types. Although different in length and amino acids composition, the effect observed on the substrate specificity was marginal when swapping the C-term flaps, like for the reaction specificity. This favours the hypothesis that the long C-term flap has low physical contact with the rest of the protein and may not play a direct role in the enzyme selectivity. Nevertheless, our results tend to show that its presence is crucial as its deletion was deleterious for the overall activity of the lipase/acyltransferase CduLAc. Interestingly, Wikmark et al[13b] showed that its deletion in CAL-A was not so damaging, but their experimental conditions were quite different from ours and the truncated sequence was slightly shorter.

Regarding the influence of the cap, the mutant CalLAc8_cap, had a specificity profile similar to the one of CduLAc. The exchange of sub-domain caused a significant increase of the specific activity for the long-chain fatty esters, particularly for C16:0, but the specific activity towards C8:0 remained the same. This confirms that the cap of CduLAc accommodates better the long chain substrates than the one of CalLAc8. This is consistent with the previous docking observations on the 3D models of the wild enzymes and shows that the acyl-donor specificity can be transferred by cap "exchange".

However, the mutant CalLAc8_cap_ct presented an increase of 1/α for the C8:0 to C12:0 EE and a decrease for the C14:0 to C18:0 EE. The specific activities for the C8:0 and C16:0 EE (0.350 and 0.160 $U.mg^{-1}$) were equivalent to the ones of CalLAc8 (0.306 $U.mg^{-1}$ and 0.147 $U.mg^{-1}$), while the specific activities for C10:0 and C12:0 EE were significantly increased (3.4 times for C10:0 EE). Therefore, it seems that combined effect of the C-term and cap is different from that of the cap only and more difficult to predict.

Influence of temperature on activity

Finally, we have evaluated the hydrolysis activities of CduLAc, CalLAc8, at various temperatures, in a range of 5 to 80°C, with ethyl oleate as substrate. To assess the influence of the subdomain change on the enzyme behavior towards temperature increase, same experiments were realized on the chimeric enzymes. Reactions were performed in buffered aqueous emulsions at pH 6.5. The experimental determination of the specific activities at each temperature allowed the calculation of the enzymes' activation energies E_A in these conditions, using the Arrhenius model.

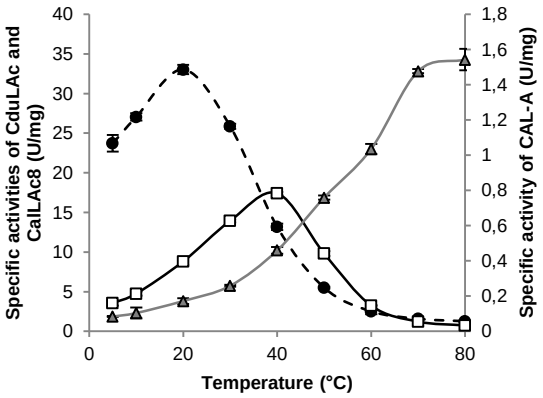


Figure 6 Influence of temperature on the hydrolysis activity of CduLAc (□), CalLAc8 (●) and CAL-A (▲). Reactions were performed during 15 min at temperatures in the range 5 – 80°C, pH 6.5 in the presence of 10 mM of ethyl oleate in PVA emulsion.

Table 3. Activation energies of CalLAc8, CduLAc, CAL-A and the chimeric enzymes for the hydrolysis of ethyl oleate at pH 6.5. Enzymes in green are non cold-active, in blue cold active and in orange thermophilic. Optimum temperatures are also provided.

Enzyme	E_A (kJ.mol^{-1})	Optimum temperature
CduLAc	34	40°C
CduLac_woC	34	40°C
CalLAc8_cap_ct	31	20°C
CalLAc8	15	20°C
CalLAc8_cap	15	20°C
CalLAc8_ct	14	20°C
CAL-A	34	> 80°C
CAL-A_ct	29	> 80°C

As presented in figure 6, CalLAc8 and CduLAc exhibited heat lability with a significant decrease of activity above 20°C and 40°C respectively, to reach 4% of their maximal activity at 80°C after 15 min of reaction. As observed before for the characterized lipases/acyltransferases CpLIP2 and CtroL4, the enzymes of this peculiar family do not seem to share the exceptional thermostability of CAL-A [5]. As shown in table 3, despite their high level of identity, CduLAc has a mesophilic profile, with an activation energy of 34 kJ.mol^{-1} while CalLAc8 is a cold-active enzyme, with E_A of 15 kJ.mol^{-1}, in the same range as the one of CpLIP2, described by Neang et al [5] .

The exchanges of cap and C-term flap did not have an impact on the cold active property of CalLAc8 (CalLAc8_cap and CalLAc8_ct) nor on the thermophilic characteristic of CAL-A (CAL-A_ct). Indeed, the activation energies of CalLAc8_cap and CalLAc8_ct were respectively of 17 kJ.mol^{-1} and 16 kJ.mol^{-1}, a same order of magnitude as the one of CalLAc8 (15 kJ.mol^{-1}). At 80°C, the activity of CAL-A_ct was still increasing, as observed with CAL-A. Regarding CduLAc_woC, the depletion of the C-term in CduLAc did not have an impact on the profile of activity at various temperatures and no change of E_A was observed. Thus, it seems that the C-term plays a crucial role in the activity of the enzyme, regardless of the temperature.

Surprisingly, the conjugated effect of the cap and C-term flap was unfavorable to the cold active property of CalLAc8 (CalLAc8_cap_ct). Indeed, as presented in table 3, the value of E_A was 31 kJ.mol^{-1} for CalLAc8_cap_ct. Thus CalLAc8_cap_ct had a mesophilic profile with medium E_A, like CduLAc. Some interactions might exist between the C-term and the cap of CduLAc increasing the rigidity of the chimer CalLAc8_cap_ct that could explain the loss of the cold active property[20].

Apart from the differences observed for CalLAc8_cap_ct, the exchange of subdomains did not change the enzymes' activity profile towards temperature. When functional, the chimeric enzymes therefore appear quite stable. It is not very surprising for CalLAc8_cap which in fact has 96% identity with CalLAc8. As the flap exchanges did not have an impact on the activity profile towards temperature, it again tends to confirm that the flap has only few contacts with the rest of the protein but its role in the enzyme activity remains unclear.

Conclusions

Despite the high level of identity (82 %) between lipases/ acyltransferases CduLAc and CalLAc8, several differences in their catalytic behavior were enlightened. Indeed CduLAc belongs to the class I of lipases/acyltransferases while CalLAc8 is a less efficient acyltransferase and is related to class II. Apart from the difference in the reaction specificity, we showed that the acyl donor preference of CduLAc is characterized by a bimodal distribution (C8 and C16 being the preferred substrates) while the one of CalLAc8 is very specific of C8. By docking experiments, we managed to identify three binding pockets for the lipidic substrate in CduLAc (two of them buried in the cap), while there were only two in CalLAc8, suggesting that the cap plays an important role in the accommodation of the substrate.

To investigate further the role of the cap and C-term flap subdomains in the catalytic behavior of the lipases/ acyltransferases, we generated chimeric enzymes based on CduLAc and CalLAc8 on one hand and CduLAc and CAL-A on the other hand. The exchanges of cap and C-term modified the activity ($r_{t\,max}$) but did not have a major impact on the acyltransfer ability ($[MeOH]_{TR50}$ and $(k_t/k_h)_{app}$. It is therefore the few differences observed in the amino acids composition of the nucleophile pocket that are probably crucial for the preference for the alcohol compared to water. The main core of the proteins, comprising the nucleophile pocket enlightened by Jan et al.[7c] was thus indirectly proven to be the most important part of the protein in the acyltransfer ability. On the other hand, the increase of $r_{t\,max}$ observed for CalLAc8_cap thus seems to be conditioned by the accommodation of the lipid substrate. This favors the hypothesis that the formation of the acyl-enzyme is the rate-limiting step of the reaction. The acyl donor specificity

seems to rely more on the cap. Indeed, the specificity of the mutant CalLAc8_cap differed from that of CalLAc8 and was more similar to the one of CduLAc. This result shows that cap engineering is a promising tool to modify that acyl-donor substrate specificity.

Further investigations have to be conducted on the role of the C-term. Indeed, if we evidenced its implication in the activity of the enzyme, we could not point out the role of this subdomain precisely. Interactions might be possible between the C-term and the cap and could be favored when both have the same origin (*e.g*. those of CduLAc). More interactions could rigidify the structure, limit the access to the active site to longer substrate (C16), and increase the stability (CalLAc8, CalLAc8_cap, CalLAc8_ct are cold-active while CalLAc8_cap_ct is not). Yet, this is not totally true for the wild CduLAc which favors the C16 substrate but is not cold active, so it is difficult to conclude. Only the comparison of real structures would allow clear elucidation of the complex structure/function relationships of such chimeric enzymes. Wikmark et al[13b] suggested that the C-term might have a role in the interfacial activation of CAL-A, as the truncated variant of this enzyme followed a Mickaelis-Menten kinetic, but their use of amphiphilic substrate up to the critical micellar concentration is not really comparable to our conditions with water/lipid interface. To show the impact of the C-term and its length on the interfacial activation, further studies are required on CduLAc and CduLAc_woC on one hand and CAL-A and CAL-A_ct on the other hand.

Experimental Section

Sequences analyses

Sequence visualization and multisequence alignments were generated using Seaview [21] and ClustalX [22]. Protein sequence identities were obtained using the SIM alignment tool for protein sequences program [23].

Evaluation of thermodynamic activities

Thermodynamic activities of water and others alcohols at 30°C in the presence of ethyl oleate (for methanol) or methyl oleate (for ethanol, propan-1-ol, propan-2-ol, butan-1-ol, butan-2-ol and isobutanol) in a two-phases system were estimated using the UNIFAC group contribution method [24] using the liquid liquid equilibrium parameters [25].

Construction and analysis of the 3D models

The 3D models of CduLAc and CalLAc8 were designed, based on the crystallographic structure of CAL-A (PDB ID: 2VEO, 3GUU)[13a] and the 3D model of CpLIP2 described by Subileau et al [7a], using Modeller 9.14[26] via UCSF Chimera 1.10.2[27]. The cavities inside the proteins were determined using the online program CASTp[28]. Docking experiments were conducted with ethyl caprylate, ethyl laurate and ethyl palmitate using AutodockVina[29] via UCSF Chimera. The score attributed to the docked molecules in Chimera can be assimilated to a binding energy. Therefore, using the Arrhenius equation, we calculated the dissociation constants (K_D) of each docked molecule of lipidic substrates.

Plasmids, strains and reagents

Competent cells of *Escherichia coli* XL1-Blue MRF' (Stratagene la Jolla, CA, USA) were used for DNA propagation. *K. pastoris* X-33, pPICZαB vector and zeocin were from Invitrogen (Life Technologies SAS, Saint Aubin, France). SacI restriction enzyme and RNaseA were purchased from Roche Diagnostic AG (Rotkreuz, Switzerland). All lipase substrates and reagents were purchased from Sigma-Aldrich (Lyon, France) and were of analytical grade.

Production of recombinant lipases

The protein sequence of CAL-A from *M. (C.) antarctica* was obtained from NCBI databases under accession number 2VEO_A. The sequences of CalLAC8 from *C. albicans* and CduLAc from *C. dubliniensis,* accessible under numbers XP_711685 and XP_002421466.1, respectively, were from Subileau et al [4a] and Jan et al [7b], with the signal peptide suppressed. DNA was synthesized and subcloned by Life Technologies (Regensburg, Germany) in pPICZαB in fusion with the signal peptide of the alpha-mating factor of *Saccharomyces cerevisiae* provided in the plasmid, as described by Neang et al [9]. To produce mutants CalLAc8_cap, CalLAc8_ct, CalLAc8_cap_ct, CduLAc_woC, CaL-A_ct and CAL-A_cap, site directed mutagenesis was performed by Life Technologies on the plasmids containing the gene of CalLAc8 (for CalLAc8_cap, CalLAc8_ct and CalLAc8_cap_ct), the gene of CduLAc (for CduLAc_woC) or the gene of CAL-A (for CAL-A_ct and CAL-A_cap). Clones for the expression of the different enzymes were produced as described by Neang et al [9]. Recombinant lipases were obtained from culture supernatants of transformed *K.pastoris* as described by Brunel et al [30] and modified by Neang et al [9].

Enzyme extract preparation

The final 800 mL culture medium was centrifuged at 4°C for 10 min at 10 000 x*g* to eliminate the cells. The supernatant (approximately 700 mL) was successively filtered with 5.0, 0.8 and 0.45 µm pores diameter filters (Millipore, Molheim, France) before its concentration to 90 mL by hollow-fiber tangential flow ultrafiltration using a Quixstand apparatus (GE Healthcare, Chalfont St Giles, UK) equipped with a 30 KDa cut-off module. Diafiltration with 600 mL of ultrapure water was then performed in the same ultrafiltration system. Enzymes were produced at multigram per liter scale.

Protein analysis

Protein concentrations in enzymatic extracts were determined by the Bradford method [31] using pure, lyophilized CpLIP2 as standard. Protein concentrations are therefore given in mg equivalent CpLIP2 protein per mL. SDS-PAGE was performed according to method of Laemmli [32] using 10% acrylamide gels. Proteins were stained with Coomassie Brilliant Blue R-250. Molecular weight were evaluated as described by Subileau et al [4a].

Enzymatic assays

Hydrolysis and transesterification (alcoholysis) activities were respectively determined by measuring the initial rates of fatty acid and ester production ($\mu mol.min^{-1}.mL^{-1}$). The transfer ratio (TR in %) corresponds to the relative initial rate of alcoholysis compared to the total activity (initial rates of alcoholysis plus competitive hydrolysis). Specific activity is the total activity ($\mu mol.min^{-1}.mL^{-1}$) divided by protein concentration ($mg.mL^{-1}$). Results are expressed as the mean of 3 independent repetitions ± standard deviation.

The amount of enzyme was adapted for each biocatalyst so as to be in conditions where the initial rate of hydrolysis of the lipidic substrate was proportional to protein concentration, in the presence of saturated or

unsaturated esters. The transesterification reaction was realized using ethyl oleate as a substrate when methanol was the acyl acceptor and using methyl oleate when acyl acceptors were other alcohols (ethanol, propan-1-ol, propan-2-ol, butan-1-ol, butan-2-ol and isobutanol). Specificity constants 1/α [18] were determined using an equimolar mixture of six saturated ethyl esters as substrates (C8:0, C10:0, C12:0, C14:0, C16:0 and C18:0, 1.6 µmol each in 1 mL of reaction medium) as previously described [18]. The influence of temperature on the enzymatic activity, was studied between 5°C and 80°C. Experiments were conducted as described by Neang et al [9] and modified by Jan et al [7b].

As described by Subileau et al [4a], the following parameters were determined from experiments with various methanol concentrations:

- The maximum specific transfer rates $r_{t\ max}$ (it has to be noted that because the enzymes studied were in-lab produced in the same system and exhibit comparable mass, the $r_{t\ max}$ can be used to compare the efficiency of the enzymes).
- The alcohol concentration required to reach 50% of transfer ratio (TR) $[MeOH]_{TR50}$,
- The ratio of the apparent rate constants for the acyltransfer to methanol and for hydrolysis $(k_t/k_h)_{app}$, determined from the initial rates for acyltransfer to methanol (r_t) and for hydrolysis (r_h) and nucleophiles concentrations ($[H_2O]$ and [MeOH]) with the following equation [17] :

$$(2)\left(\frac{k_t}{k_h}\right)_{app} = \frac{r_t}{r_h}\cdot\frac{[H_2O]}{[MeOH]}$$

A linear relationship was observed between the r_t/r_h and the $[MeOH]/[H_2O]$ ratios within the range of methanol concentrations tested, with $(k_t/k_h)_{app}$ as the slope. $(k_t/k_h)_{app'}$ is also the ratio of the apparent rate constants of acyltransfer versus hydrolysis but determined as the slope of the initial rates ratio r_t/r_h as a function of the ratio of the corresponding methanol versus water thermodynamic activities a_{MeOH}/a_W.

Acknowledgements

Authors thank the University of Montpellier and Montpellier SupAgro for the PhD granting of Ms. AH Jan.
GC analyses were performed using LiPolGreen platform (http://www.supagro.fr/plantlippol-green/).
K. pastoris cultivations were conducted on the UMR IATE fermentation platform, a member of the I-FERM consortium (http://www.i-ferm.com/en).

Keywords: lipases/acyltransferases • synthesis *de novo* • structure/function • cap • chimeric enzymes

References

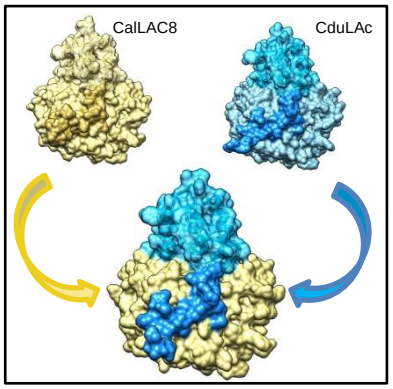
[1] U. Schörken, P. Kempers, *Eur J Lipid Sci Technol* **2009**, *111*, 627-645.
[2] a) F. Hasan, A. A. Shah, A. Hameed, *Enzyme Microb Tech* **2006**, *39*, 235-251; b) P. Villeneuve, *Biotechnol Adv* **2007**, *25*, 512-536.
[3] L. Casas-Godoy, S. Duquesne, F. Bordes, G. Sandoval, A. Marty, *Methods Mol Biol* **2012**, *861*, 3-30.
[4] a) M. Subileau, A. H. Jan, J. Drone, C. Rutyna, V. Perrier, E. Dubreucq, *submitted in RSC Catal. Sci. Tech.* **2016**, *CY-ART-08-2016-001805*; b) K. Zorn, I. Oroz-Guinea, H. Brundiek, U. T. Bornscheuer, *Prog Lip Res* **2016**, *63*, 153-164.
[5] P. M. Neang, M. Subileau, V. Perrier, E. Dubreucq, *Appl Microbiol Biotechnol* **2014**, *98*, 8927-8936.
[6] D. Briand, E. Dubreucq, P. Galzy, *Biotechnol Lett* **1994**, *16*, 813-818.
[7] a) M. Subileau, A. H. Jan, H. Nozac'h, M. Pérez-Gordo, V. Perrier, E. Dubreucq, *BBA Proteins Proteom* **2015**, *1854*, 1400-1411; b) A. H. Jan, M. Subileau, C. Deyrieux, V. Perrier, E. Dubreucq, *BBA Proteins Proteom* **2016**, *1864*, 187-194; c) A. H. Jan, D. E., M. Subileau, *submitted in ASC Catal.* **2016**, *cs-2016-02883v*.
[8] M. Fischer, J. Pleiss, *Nucleic Acids Res* **2003**, *31*, 319-321.
[9] P. M. Neang, M. Subileau, V. Perrier, E. Dubreucq, *J Mol Catal B: Enzym* **2013**, *94*, 36-46.
[10] E. Husson, C. Humeau, C. Harscoat, X. Framboisier, C. Paris, E. Dubreucq, I. Marc, I. Chevalot, *Process Biochem.* **2011**, *46*, 945-952.
[11] L. Vaysse, E. Dubreucq, J.-L. Pirat, P. Galzy, *J Biotechnol* **1997**, *53*, 41-46.
[12] M. Widmann, P. B. Juhl, J. Pleiss, *BMC Genomics* **2010**, *11*, 123.
[13] a) D. J. Ericsson, A. Kasrayan, P. Johansson, T. Bergfors, A. G. Sandstrom, J. E. Backvall, S. L. Mowbray, *J Mol Biol* **2008**, *376*, 109-119; b) Y. Wikmark, K. Engelmark Cassimjee, R. Lihammar J. E. Backvall, *Chembiochem* **2016**, *17*, 141-145.
[14] a) S. Brocca, F. Secundo, M. Ossola, L. Alberghina, G. Carrea, M. Lotti, *Protein Sci* **2003**, *12*, 2312-2319; b) C. C. Akoh, G. C. Lee, J. F. Shaw, *Lipids* **2004**, *39*, 513-526.
[15] F. Secundo, G. Carrea, C. Tarabiono, P. Gatti-Lafranconi, S. Brocca, M. Lotti, K. E. Jaeger, M. Puls, T. Eggert, *J Mol Catal B-Enzym* **2006**, *39*, 166-170.
[16] M. Skjot, L. De Maria, R. Chatterjee, A. Svendsen, S. A. Patkar, P. R. Ostergaard, J. Brask, *Chembiochem* **2009**, *10*, 520-527.
[17] V. Kasche, *Enzyme Microb Tech* **1986**, *8*, 4-16.
[18] L. Vaysse, A. Ly, G. Moulin, E. Dubreucq, *Enzyme Microb Tech* **2002**, *31*, 648-655.
[19] a) M. Norin, F. Haeffner, A. Achour, T. Norin, K. Hult, *Protein Sci* **1994**, *3*, 1493-1503; b) M. Holmquist, *Chem Phys Lipids* **1998**, *93*, 57-66; c) J. Pleiss, M. Fischer, R. D. Schmid, *Chem Phys Lipids* **1998**, *93*, 67-80; d) P. Grochulski, F. Bouthillier, R. J. Kazlauskas, A. N. Serreqi, J. D. Schrag, E. Ziomek, M. Cygler, *Biochemistry* **1994**, *33*, 3494-3500.
[20] C. Struvay, G. Feller, *Int J Mol Sci* **2012**, *13*, 11643-11665.
[21] N. Galtier, M. Gouy, C. Gautier, *Comput Appl Biosci* **1996**, *12*, 543-548.
[22] M. A. Larkin, G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm, R. Lopez, J. D. Thompson, T. J. Gibson, D. G. Higgins, *Bioinformatics* **2007**, *23*, 2947-2948.
[23] X. Huang, *Adv Appl Math* **1991**, *12*, 337-357.
[24] A. Fredenslund, R. L. Jones, J. M. Prausnitz, *AIChE J* **1975**, *21*, 1086-1099.
[25] a) T. Magnussen, P. Rasmussen, A. Fredenslund, *Ind Eng Chem Process Des Dev* **1981**, *20*, 331-339; b) P. Oracz, M. Goral, *J Chem Eng Data* **2011**, *56*, 4853-4861.
[26] a) A. Fiser, R. K. Do, A. Sali, *Protein Sci* **2000**, *9*, 1753-1773; b) M. A. Marti-Renom, A. C. Stuart, A. Fiser, R. Sanchez, F. Melo, A. Sali, *Annu Rev Biophys Biomol Struct* **2000**, *29*, 291-325; c) A. Sali, T. L. Blundell, *J Mol Biol* **1993**, *234*, 779-815.
[27] E. F. Pettersen, T. D. Goddard, C. C. Huang, G. S. Couch, D. M. Greenblatt, E. C. Meng, T. E. Ferrin, *J Comput Chem* **2004**, *25*, 1605-1612.
[28] J. Dundas, Z. Ouyang, J. Tseng, A. Binkowski, Y. Turpaz, J. Liang, *Nucleic Acids Res* **2006**, *34*, W116-118.
[29] O. Trott, A. J. Olson, *J Comput Chem* **2010**, *31*, 455-461.
[30] L. Brunel, V. Neugnot, L. Landucci, H. Boze, G. Moulin, F. Bigey, E. Dubreucq, *J Biotechnol* **2004**, *111*, 41-50.
[31] M. M. Bradford, *Anal Biochem* **1976**, *72*, 248-254.
[32] U. K. Laemmli, *Nature* **1970**, *227*, 680-685.

10.1002/cbic.201600672

WILEY-VCH

FULL PAPER

Design of chimeric enzymes: Despite 82% identity, the 2 recently discovered lipases/acyltransferases, CalLAc8 and CduLAc displayed different catalytic behavior. To elucidate the role of the subdomains (main core, cap and C-term flap) of these enzymes, chimeric enzymes were designed and characterized. This evidenced the implication of the cap in the substrate specificity and the main core in the acyltransfer ability.



*A-H. Jan, E. Dubreucq, M. Subileau**

Page No. – Page No.

Enlightening the role of subdomains in the catalytic behavior of lipases/acyltransferases homologous to CpLIP2 through rational design of chimeric enzymes