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To cite this version:

Pisey M Neang, Maeva Subileau, Véronique Perrier, Eric Dubreucq. Homologous yeast lipases/acyltransferases exhibit remarkable cold-active properties.. Applied Microbiology and Biotechnology, 2014, 98 (21), 10 p. 10.1007/s00253-014-5776-6 . hal-01189940

HAL Id: hal-01189940

https://hal.science/hal-01189940v1

Submitted on 1 Sep 2015

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Appl Microbiol Biotechnol
DOI 10.1007/s00253-014-5776-6

BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Homologous yeast lipases/acyltransferases exhibit remarkable cold-active properties

Pisey M. Neang · Maeva Subileau · Véronique Perrier · Eric Dubreucq

Received: 30 January 2014 / Revised: 10 April 2014 / Accepted: 12 April 2014
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Abstract Lipases/acyltransferases catalyse acyltransfer to various nucleophiles preferentially to hydrolysis even in aqueous media with high thermodynamic activity of water (a_w >0.9). Characterization of hydrolysis and acyltransfer activities in a large range of temperature (5 to 80 °C) of secreted recombinant homologous lipases of the *Pseudozyma antarctica* lipase A superfamily (CaLA) expressed in *Pichia pastoris*, enlighten the exceptional cold-activity of two remarkable lipases/acyltransferases: CpLIP2 from *Candida parapsilosis* and CtroL4 from *Candida tropicalis*. The activation energy of the reactions catalysed by CpLIP2 and CtroL4 was 18–23 kJ mol^{-1} for hydrolysis and less than 15 kJ mol^{-1} for transesterification between 5 and 35 °C, while it was respectively 43 and 47 kJ mol^{-1} with the thermostable CaLA. A remarkable consequence is the high rate of the reactions catalysed by CpLIP2 and CtroL4 at very low temperatures, with CpLIP2 displaying at 5 °C 65 % of its alcoholysis activity and 45 % of its hydrolysis activity at 30 °C. These results suggest that, within the CaLA superfamily and its homologous subgroups, common structural determinants might allow both acyltransfer and cold-active properties. Such biocatalysts are of great interest for the efficient synthesis or functionalization of temperature-sensitive lipid derivatives, or more generally to lessen the environmental impact of biocatalytic processes.

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Keywords Cold-active enzyme · Alcoholysis · Lipase/acyltransferase · *Pseudozyma antarctica* lipase A · *Candida parapsilosis* · *Candida tropicalis*

Introduction

Lipases (EC 3.1.1.3) are ubiquitous enzymes that naturally catalyse hydrolysis of fats and oils into free fatty acids and glycerol. In addition to hydrolysis, they are able to catalyse condensation reactions such as esterification or acyltransfer reactions like alcoholysis. The diversity of their origins (plants, animals and microorganisms) ensures not only their natural availability but also their huge varieties of functional characteristics for applications in diverse sectors (Jaeger and Eggert 2002; Hills 2003; Hasan et al. 2006; Villeneuve 2007; Christopher et al. 2014). Because the presence of water in the reaction is generally favourable to hydrolysis, the use of lipases for synthesis reaction is mostly conducted in media with strict control of the a_w (in organic solvent or solvent-free conditions) in which enzyme stability is crucial (Adlercreutz 2013; Christopher et al. 2014). The development of such processes thus becomes quite challenging when two substrates with different polarity and solvent affinity are to be reacted (Villeneuve 2007). Besides, one of the most important characteristic for industrial enzyme development is their operational stability. Although it is generally considered that industrial applications require thermostable enzymes, heat-labile, cold-adapted lipases have been increasingly used in a wide range of applications, especially the detergents (cold washing), food industries (fermentation, cheese manufacture, meat tenderizing, lipid structuration), environmental bioremediations, biotransformation and molecular biology (Gerday et al. 2000; Joseph et al. 2008). Cold-active enzymes exhibit high catalytic activity at near-zero to room temperatures because of a higher flexibility and/or a local instability that allow

Published online: 29 April 2014

the reduction of the free energy barrier of the transition state by destabilizing the structures bearing the active site or the whole molecule (decrease of activation enthalpy, increase conformational entropy) (Cavicchioli et al. 2002; Roca et al. 2007; Casanueva et al. 2010; Feller 2010; Struvay and Feller 2012). Cold-active lipases are thus of particular interest for the lipophilization and production of thermolabile compounds at high rates and for the economic benefits through energy saving: functioning in/during cold environments/season, minimizing undesirable higher temperature occurring-chemical reactions, avoiding the requirement of unnecessary heating steps and being rapidly and easily inactivated when required (Gerday et al. 2000; Joseph et al. 2008). These lipases are usually produced by psychrophilic microorganisms isolated from cold environments such as the Antarctic (Parra et al. 2008; Wang et al. 2012; Litantra et al. 2013), deep-sea sediments (Jeon et al. 2009) or other cold regions of the world (Zheng et al. 2011; Tanaka et al. 2012). In order to survive and proliferate in such extreme conditions, these microorganisms must synthesize naturally evolved enzymes exhibiting high activity at low temperature. Several studies have brought insights into protein structure and temperature adaptation relationships. It is generally admitted that a higher flexibility remains the main adaptive characteristic of psychrophilic enzymes compared to their meso- or thermophilic homologs. Within proteins families, different structural strategies exist to reach this structural specificity (Nielsen et al. 1999; Gianese et al. 2002; Siddiqui and Cavicchioli 2006; Joseph et al. 2008; Struvay and Feller 2012; Florczak et al. 2013). In literature, the major structural determinants described in cold-active enzymes are a decrease of the number of the ion pairs and salt bridges (reduced number of aspartic acid, glutamic acid and arginine residues), the charge-dipole interactions in α-helices and side-chain contributions to the exposed surface, the apolar fraction of the buried surface and the aromatic-aromatic interactions, all consistent with a decrease of optimum temperatures (Arpigny et al. 1997; Smalås et al. 2000; Gianese et al. 2002; Joseph et al. 2008; Casanueva et al. 2010). These subtle structural characteristics are often localized close to the catalytic site or the specificity pocket, but also in regions not directly involved in catalysis, in loops and/or mobile subparts (lids) (Wolf-Watz et al. 2004; Papaleo et al. 2008). It has been proposed that the high flexibility of cold-active enzymes facilitates the positioning of the substrate in the active site at low energy cost, resulting in high specific activities but also generally in heat lability and low substrate affinity (Lonhienne et al. 2000; Cavicchioli et al. 2002; Siddiqui and Cavicchioli 2006; Roca et al. 2007; Feller 2010; Struvay and Feller 2012).

Cold-active lipases have been mostly identified in psychrophilic organisms, yet some have also been characterized from mesophilic yeasts such as *Pichia lynferdii* (Kim et al. 2010; Park et al. 2013) and *Candida albicans* (Lan et al. 2011). Interestingly, the cold-adapted lipase CaLIP5 from *C. albicans* (Lan et al. 2011) can be classified into the superfamily of the thermostable CaLA lipase from *Pseudozyma (Candida) antarctica* (Fig. 1), both enzymes exhibiting 34 % of identity in their primary sequence. In spite of the original habitat of the strain, *P. antarctica* was shown to grow in 5–32 °C temperature range (Goto et al. 1969), and the enzyme CaLA has been shown to exhibit exceptionally high thermal stability, allowing applications at temperatures superior to 90 °C (Heldt-Hansen Hans et al. 1989; Domínguez de María et al. 2005). CaLA has be extensively studied (Sandstrom et al. 2009; Engström et al. 2010; Naik et al. 2010; Brenneis and Baeck 2012; Sandstrom et al. 2012), and homologous enzymes are considered to constitute an original group of lipases, with low structural homologies with other characterized lipases (Ericsson et al. 2008; Kourist et al. 2010; Widmann et al. 2010; Brundiek et al. 2012).

The present work aimed at studying the effect of a large range of temperatures (5–80 °C) on the hydrolysis and alcoholysis activities in bi-phasic aqueous medium (lipid as organic phase, emulsified in buffer as aqueous phase) of four recombinant lipases of the CaLA superfamily, heterologously produced in *Pichia pastoris*: CpLIP2 from *Candida parapsilosis* CBS 604, CtroL4 from *Candida tropicalis* MYA-3404, AflaL0 from *Aspergillus flavus* NRRL3357 and CaLA (Neang et al. 2013). In similar experimental conditions, our group has already shown that CpLIP2 and CtroL4 exhibited an exceptional acyltransferase activity (Briand et al. 1995a; Neang et al. 2013) and thus appear as perfect candidates for the development of greener biocatalytic processes compare to those conducted in organic solvents. Indeed, compared to "classical" lipases, these two lipases/acyltransferases catalyse acyltransfer to various nucleophiles (e.g. alcoholysis reaction) preferentially to hydrolysis even in aqueous media with high thermodynamic activity of water (a_w >0.9), resulting in a kinetically controlled synthesis (Lecointe et al. 1996; Gianese et al. 2002, 2002; Neang et al. 2013) and offering great potential for example for the lipophilization of polar substrates (Villeneuve 2007; Husson et al. 2011). The catalysed reaction indeed results in a transesterification ratio superior to that obtained with the thermodynamic equilibrium of the esterification reaction. The interest of such kinetically controlled reactions has been described for acyltransfer catalysis in water abundant media, such as for the synthesis of peptides bonds using proteases or for amine-acylation catalysed by penicillin-acylases (Riechmann and Kasche 1984, 1985; Kasche 1986; Morihara 1987; Gololobov et al. 1993; Guranda et al. 2001; Chilov et al. 2003; Kumar and Bhalla 2005; Bahamondes et al. 2012).

The four lipases presented here exhibit 31 to 58 % of identity in their primary sequences, and even if CaLA is currently the only one with a 3D structure elucidated by X-ray diffraction (Ericsson et al. 2008), our goal was also to contribute through functional characterizations to a better

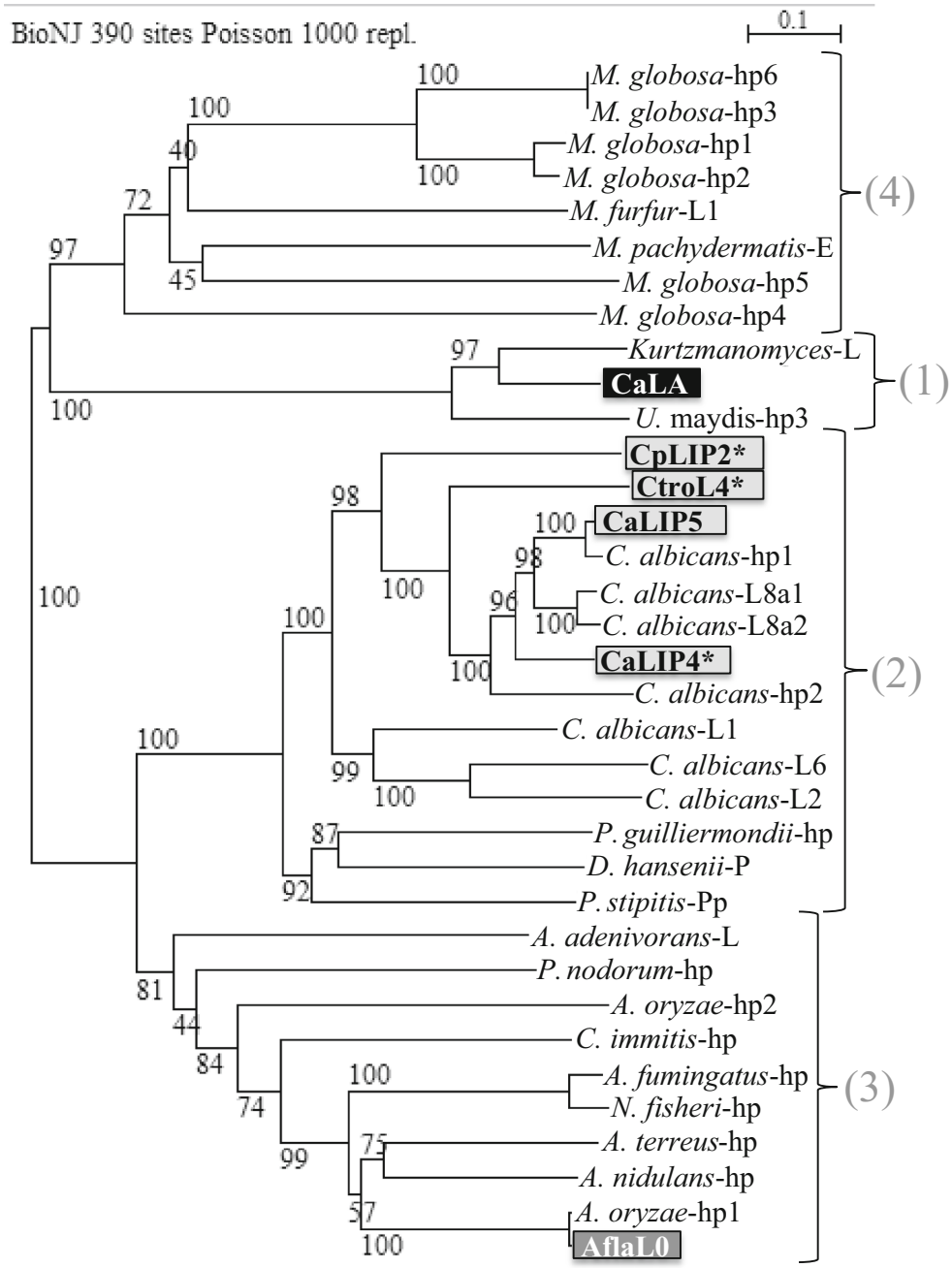

Fig. 1 Distance tree of the proteins of the CaLA superfamily, with its four families as described in the Lipase Engineering Database: (1) *C. antarctica* lipase A like; (2) *C. albicans* lipase like; (3) *Aspergillus* lipase like; (4) *Malassezia* lipase like. Enzymes highlighted in *light grey*, *dark grey* and *black* are respectively cold-adapted, mesophilic and thermophilic; *Asterisk* (*) *enzymes with acyltransferase ability demonstrated in aqueous media*

understanding of the various specificities and thermostability exhibited by such homologous proteins and to provide data to the homology models currently under study in our group.

Materials and methods

Sequence analysis

Proteins sequences of the CaLA superfamily were obtained from the Lipase Engineering Database (Fischer and Pleiss 2003). Sequence visualization, multisequence alignments and distance trees (BioNJ, Poisson, bootstrap 1000) were generated using Seaview (Galtier et al. 1996). Protein families are organized on the level of homologous families and superfamilies based on their sequence similarity. Protein sequence identity was obtained using the SIM alignment tool for protein sequences program (Huang and Miller 1991).

Recombinant enzymes

Recombinant CpLIP2, CtroL4, AflaL0 and CaLA were obtained from culture supernatant of transformed *P. pastoris* as previously described by Brunel et al. (2004) and Neang et al.

(2013). Briefly, cultivations were performed using a synthetic medium containing only minerals, biotin, glycerol or methanol (carbon and energy source) and ammonia (nitrogen source). At the end of the culture, the enzyme extracts were first concentrated by ultrafiltration and then submitted to a diafiltration against pure water, so that no significant amount of minerals or low molecular mass compounds was present in the final extract (cut-off 30,000 Da).

Enzymatic assays

Hydrolysis and transesterification (or "acyltransfer" or "alcoholysis") activities were respectively determined by measuring the initial rates of fatty acid and of ester production (μmol min^{-1}) in aqueous emulsions as described below. Specific activities were calculated in relation to protein concentration. The transesterification ratio is the percentage of alcoholysis rate vs the total activity rate (hydrolysis + alcoholysis) in the same conditions (in the presence of 2.2 M methanol in this study). Results are expressed as the mean of three independent repeats ± standard deviation.

Experiments were conducted as previously described (Vaysse et al. 2002; Neang et al. 2013) with the following modifications. Lipid substrates were prepared as emulsions consisting of 100 mM lipid emulsified by sonication (Branson Sonifier 250, 20 s, 200 W, 145 μm amplitude) in an aqueous solution of 20 g L^{-1} poly(vinyl alcohol). For reactions (total volume 1 mL in stoppered glass tubes), 100 μL of the lipid substrate emulsion were added to 800 μL of 50 mM sodium phosphate buffer, pH 6.5, eventually containing 2.2 M methanol for transesterification assays. After 45 s of preheating of the reaction medium, reactions were started by the addition of 100 μL of enzyme solution in the same buffer. The amount of enzyme was adapted for each biocatalyst so as to be in conditions where the rate of hydrolysis of ethyl oleate was detectable, proportional to protein concentration and constant during 15 min (0.002, 0.002, 0.13 and 0.115 mg proteins (eq. CpLIP2) in 1 mL of reaction medium for CpLIP2, CtroL4, CaLA and AflaL0, respectively). After 15 min, reactions were stopped by the addition of 950 μL of an ethanol/sulphuric acid (100:0.8, *v*/*v*) mixture. After the addition of 50 μL of internal standards (ethanolic solution of pentadecanoic acid and its ethyl and methyl esters, 1 μmol each), lipids were extracted with 1 mL of hexane. Esters and free fatty acids were then analysed by gas chromatography (GC) as follows: Hexane extracts (200 μL) were reacted with 25 μL pyridine and 25 μL *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) as silylating agent. After heating at 50 °C for 20 min, samples were ready for analysis. A Hewlett-Packard 5890 GC system equipped with a flame ionization detector, an automatic sampler (sample volume 0.5 μL) and a split/splitless injector was used for analysis. The capillary column was a DB-5ht (15 m× 0.25 mm, J&W Scientific, Massy, France). The helium carrier flow was 2 mL min^{-1} and the split ratio was 1:18. Temperature conditions were, for reactions with individual substrates: injector 280 °C, detector 290 °C, oven 200 to 225 °C at 10 °C min^{-1}.

Calibration curves were realized using emulsions of mixtures of FA and monoesters prepared according to the same protocol, without enzyme and alcohol.

Protein analysis

Protein concentrations in enzymatic extracts were determined by the Bradford method (Bradford 1976) using pure, lyophilized CpLIP2 as standard. Results are therefore given in milligrams equivalent of CpLIP2. As enzyme extracts were obtained from a synthetic cultivation medium then diafiltrated against pure water, no interference could be expected between the Bradford assay and peptides or salts.

Results

The catalytic activities of CpLIP2, CtroL4, AflaL0 and CaLA in a temperature range of 5 to 80 °C were evaluated in parallel for both hydrolysis and alcoholysis of ethyl oleate (C18:1EE). Reactions were performed in buffered aqueous emulsions at pH 6.5 with or without 2.2 M methanol. The pH chosen was in the optimal activity range (>85 % of the maximal activity) at 30 °C for the four enzymes (data not shown).

First, the four enzymes exhibited significant differences of specific activities and transesterification ratios. CpLIP2 and CtroL4 exhibited exceptionally high specific activities (respective maxima of 81–88 and 86–100 μmol min^{-1}) and transesterification ratios (respective average transesterification ratios of 97 and 87 %) compared to CaLA and AflaL0 (respectively 3–3.5 μmol min^{-1} and 57 % of transesterification and 1.3–1.5 μmol min^{-1} and 46 % of transesterification). These results confirm the exceptional level of specific activities and transesterification ratios obtained with CpLIP2 and CtroL4 in our previous study (Neang et al. 2013). With CpLIP2 and CtroL4, the transesterification ratios were only slightly affected by temperature in the presence of 2.2 M methanol (less than 3 % variation), while with CaLA, it increased with the increase of temperature (from 46 % at 5 °C to 67 % at 80 °C), and with AflaL0, more variations were observed (38 to 52 %). Three behaviours were observed among the four enzymes in the conditions of temperature variation tested (Fig. 2):

(1) For CpLIP2 and CtroL4, the highest specific activity was observed between 35 and 45 °C in the absence of methanol and around 30 °C in the presence of 2.2 M methanol. No activity was detected at 60 °C and higher. Below 30 °C, the reaction rates decreased slowly with

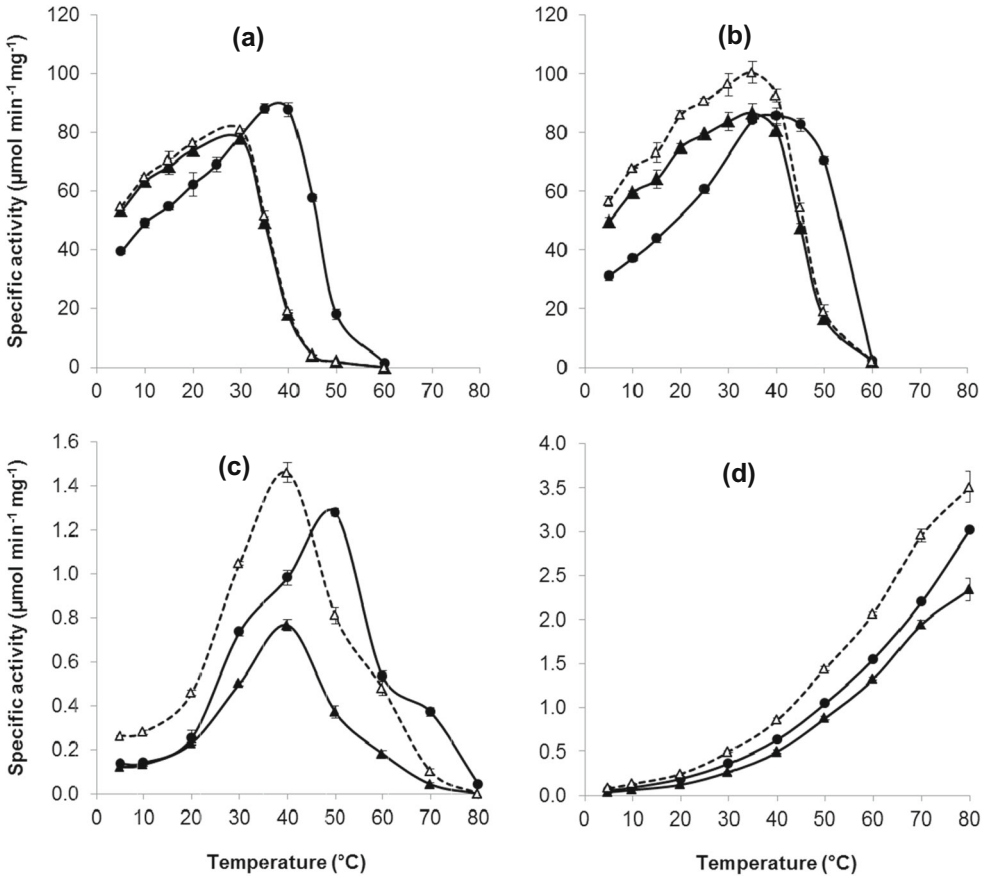

Fig. 2 Influence of temperature on catalytic activity of enzymes CpLIP2 (**a**), CtroL4 (**b**), AflaL0 (**c**) and CaLA (**d**) in condition without alcohol (*black-filled circles* (●) hydrolysis activity) and in transesterification condition with MeOH (*black-filled triangle* (▲)alcoholysis activity; *white triangle* (△) total activity (=alcoholysis + competitive hydrolysis)). Reactions were performed at desired temperature, pH 6.5 in the presence of 10 mM of ethyl oleate in PVA emulsion, and eventually 2.2 M methanol

decreasing temperatures, with both enzymes preserving a high catalytic activity at 5 °C (>30 % of the maximum, up to 65 % for CpLIP2 in alcoholysis conditions).

(2) For AflaL0, the highest rates of transesterification and hydrolysis were observed between 40 and 50 °C in the conditions tested. Above 50 °C, the reaction rates decreased regularly with temperature up to 80 °C, where no activity could be detected. At 5 °C, the specific activity of AflaL0 was only 20 % of its maximal value (i.e. at 50 °C for hydrolytic conditions and 40 °C in the presence of methanol).

(3) With CaLA, the specific activity continuously increased up to the maximum temperature tested (80 °C), which confirms the exceptional thermophilic characteristics of CaLA previously described (Bosley et al. 1997; Nielsen et al. 1999; Lund et al. 2000), even in biphasic aqueous media. In comparison, CpLIP2, CtroL4 and AflaL0 were thus less thermostable, even if, at 60 °C, AflaL0 still exhibited 24 and 42 % of its respective acyltransfer and hydrolysis maximal activities.

The presence of 2.2 M methanol seemed to have a negative impact on the stability of the four enzymes (although only a tendency can be observed for CaLA at 65–80 °C), leading to a 5–10 °C lower value of the temperature at which the highest reaction rates were recorded (Fig. 2). Indeed, methanol allowed high levels of catalytic activity at moderate temperatures, but appeared, at high temperature, to have a destabilizing effect and to be favourable to enzyme denaturation.

The relatively low thermostability of CpLIP2 and CtroL4 was counterbalanced by interesting cold-active properties. Indeed, at 5 °C, CpLIP2 and CtroL4 preserved respectively 68 and 57 % of their maximum acyltransfer activity (78 µmol min^{-1} mg^{-1} at 30 °C for CpLIP2 and 87 µmol min^{-1} mg^{-1} at 35 °C for CtroL4) and 45 and 36 % of their optimum hydrolysis activity (88 µmol min^{-1} mg^{-1} at 40 °C for CpLIP2 and 86 µmol min^{-1} Mg^{-1} at 40 °C for CtroL4). At 5 °C, AflaL0 and CaLA only preserved respectively 10–16 and 2-4 % of their activities compared to the highest activities (obtained at 40–50 °C for AflaL0 and at 80 °C for CaLA). Comparison of the specific activities of the four enzymes at 5 and 30 °C confirmed that CpLIP2 was the most cold-active lipase (with residual activities of 55 and 68 % at 5 °C compared to those obtained respectively in hydrolysis and alcoholysis condition at 30 °C), followed by CtroL4 (48–59 % of residual activity) while at 5 °C AflaL0 and CaLA only conserved respectively 19–25 and 16 % of their total activity at 30 °C.

Activation energy E_A of the reactions, calculated on the exponential parts of the curves (Fig. 2) using the Arrhenius

model, showed that CpLIP2 and CtroL4 exhibited remarkable low $E_{A,}$ especially for the alcoholysis reaction (Table 1). Indeed, compared to the classical lipases CaLA and AflaL0, for which the average E_A was 43 kJ mol^{-1}, CpLIP2 and CtroL4 exhibited an average E_A of 17 and 18 kJ Mol^{-1}, respectively. For both enzymes, the activation energy of the transesterification reaction (~15 kJ mol^{-1}) was lower than that of the hydrolysis reaction (17–23 kJ mol^{-1}). Combined to their high specific activity, these results confirmed that CpLIP2 and CtroL4 are highly efficient cold-active lipases/acyltransferases.

Discussion

Like previously described, in the presence of methanol, CpLIP2 and CtroL4 displayed an exceptional level of acyltransferase activity compared to CaLA and AflaL0, with average transesterification ratios (alcoholysis vs alcoholysis+hydrolysis in the same conditions) of 97, 87, 57 and 46 %, respectively, in the presence of 2.2 M methanol. These transesterification ratios were only slightly influenced by the reaction temperature, but acyltransfer was favoured by decreasing temperature as the maximum transesterification rates (with alcohol) were obtained at lower temperature than the maximum hydrolysis rates (without alcohol), in accordance with temperature influence proposed by Kasche (1986). In our experiments, the water phase, containing the enzyme and eventually alcohol (methanol), exhibited high water activity (a_w >0.9) and was simply emulsified with the lipid substrate (ethyl oleate in poly(vinyl alcohol) as emulsifier, without organic solvent). When methanol was present, three reactions were possible (in initial conditions): (1) production of oleic acid by hydrolysis of ethyl oleate, (2) production of methyl oleate by alcoholysis (or "acyltransfer" or "transesterification") of ethyl oleate and (3) production of methyl oleate by esterification of the firstly produced oleic acid. According to our former studies, it is was proposed that the competition between water and the other nucleophile as acyl acceptor group was modulated by both the chemical reactivity of the competitive reactants, through their relative thermodynamic activity and by intrinsic properties of the biocatalysts that will preferentially catalyse either hydrolysis or alcoholysis reactions in the deacylation step of the catalytic mechanism (Briand et al. 1995a, b; Lecointe et al. 1996; Vaysse et al. 2002; Neang et al. 2013), as described for other lipases (Bousquet-Dubouch et al. 2001; Ma et al. 2002; Graber et al. 2003a; Graber b; Adlercreutz 2013). Here, the slight differential effect of temperature on alcoholysis and competitive hydrolysis observed could not be explained by an effect of temperature on the thermodynamic activities of alcohol and water. UNIFAC calculations (Magnussen et al. 1981) suggest indeed a ~2 % increase of the thermodynamic activity of methanol with almost constant water activity when temperature raises from 5 and 30 °C in the conditions of our experiments. Therefore, it was mainly the intrinsic properties of the biocatalysts that either determined a kinetically controlled synthesis with a superior transesterification ratio obtained with the lipases/acyltransferases CpLIP2 and CtroL4, or an equilibrium control synthesis with an inferior transesterification ratio obtained with the classical lipases CaLA and AflaL0 (in theory ~70 % alcoholysis at the thermodynamic equilibrium at 2.2 M methanol).

In addition, we showed that CpLIP2 and CtroL4 displayed exceptionally low activation energy, particularly for the alcoholysis reaction. Low values of E_A are typical of cold-active lipases from different organisms such as *Psychrobacter* sp. (Parra et al. 2008), Antarctic *Bacillus pumilus* strains (Litantra et al. 2013) and *Photocacterium* strain (Kim et al. 2012), which displayed E_A of 23, 18 and 11.3 kJ mol^{-1}, in the conditions of the respective studies. In comparison, CaLIP5, the previously identified cold-active lipase belonging to the CaLA superfamily, showed higher activation energy for hydrolysis (36 kJ mol^{-1}), in a temperature range of 5–25 °C above which its enzymatic rate decreased (Lan et al. 2011). The other homologous lipase CaLIP4 (60 % homology with CpLIP2), studied in a temperature range of 20–50 °C, was shown to exhibit comparable E_A to CpLIP2 and CtroL4 for the transesterification reaction (18 kJ mol^{-1}) but significantly higher E_A for hydrolysis alone (46 kJ mol^{-1}) (Roustan et al. 2005).

Besides, CpLIP2 and CtroL4, very active at 5 °C, were nearly totally inactivated at 60 °C while AflaL0 and CaLA displayed very limited activities at low temperature. These results suggest that cold-active properties and thermostability of this group of enzymes are mutually exclusive, which is not always the case (Fedøy et al. 2007; Florczak et al. 2013). The thermolability of cold-adapted lipases is well known in literature (Feller et al. 1991; Gerday et al. 2000), and most cold-evolved lipases typically lose their hydrolysis activity at temperature higher than 25–35 °C (Kim et al. 2010; Lan et al.

Table 1 Activation energy E_A for each catalytic reaction (hydrolysis in condition without methanol, transesterification and competitive hydrolysis in the presence of 2.2 M methanol) at a temperature range of 5–35 °C in the presence of each of the four enzymes tested: CpLIP2, CtroL4, CaLA and AflaL0

Activation energy (kJ mol^{-1})				
Enzyme	Hydrolysis	Alcoholysis	Competitive hydrolysis	Mean ± standard deviation
CpLIP2	17.5	14.5	18.1	16.7±1.8
CtroL4	22.6	14.7	16.6	18.0±1.6
CaLA	43.0	47.1	40.2	43.5±3.5
AflaL0	44.4	44.6	40.9	43.3±2.1

2011; Tanaka et al. 2012; Litantra et al. 2013), including CaLIP5 for which the optimum hydrolysis activity was observed at 25 °C (Lan et al. 2011). In comparison, the apparent higher stability of CpLIP2 and CtroL4 (optimum at 30–40 °C in the conditions tested here) could be particularly efficient for specific applications at ambient temperature (no need for cooling or heating). Besides, improvement of these biocatalysts stability could be achieved by enzymes immobilization (Iyer and Ananthanarayan 2008; Cowan and Fernandez-Lafuente 2011; Garcia-Galan et al. 2011; Hwang and Gu 2013; Rodrigues et al. 2013) as it has already been shown for CpLIP2 (Osorio et al. 2009a).

The functional characterization of different members of the same protein superfamily is an interesting approach for a better understanding of the specificities of this group and its subgroups. Here, we demonstrate that, within the CaLA superfamily, cold-active enzymes are found within the *C. albicans* lipase-like family (Widmann et al. 2010) (Fig. 1), which exhibit more than 58 % mutual identity in their primary sequences. In addition, acyltransfer ability and cold activity could be related to common structural determinants shared by CpLIP2, CtroL4 and CaLIP4, the best currently described acyltransferases of the superfamily. This might be related to the higher flexibility of these proteins which might favour the positioning of larger nucleophiles than water in the active site (the lower the E_A, the higher the transesterification rate). Furthermore, the particularly high specific activities exhibited by CpLIP2 and CtroL4 also support the proposition that the high flexibility and/or local instability of cold-active enzymes could enable an increased complementarity between the active site and the substrate transition state with a low activation barrier, resulting in high specific activities at low temperatures (Cavicchioli et al. 2002; Roca et al. 2007). Classical sequence-based features acting on flexibility such as the proportion of arginine vs lysine residues, the number of proline, hydrophobic or charged residues have not been evidenced compared to their thermophilic biocatalysts. However, because the four enzymes described here are homologous, further elucidation and comparisons of their 3D structures should lead path for the design of valuable thermostable lipase/acyltransferases.

In conclusion, after investigating the ability of CpLIP2, CtroL4, AflaL0 and CaLA to catalyse both hydrolysis and alcoholysis reactions in aqueous medium (a_w >0.9) at extreme conditions of temperature, CpLIP2 and CtroL4 were shown to be cold-active lipases/acyltransferases, which displayed at 5 °C, 45–68 and 35-57 % of their optimum activities obtained at 30–40 °C. To the contrary, AflaL0 and CaLA were only slightly active at 5 °C and displayed a maximum specific activity at higher temperatures (40–50 °C for AflaL0 and higher than 80 °C for CaLA). The activity of CaLA increased steadily with the increase of temperature from 5 to 80 °C, illustrating its already known exceptional thermostability. It is interesting to note that contrary to most previously described cold-adapted enzymes, which are from psychrophilic microorganisms; CpLIP2 and CtroL4 are produced by mesophilic *C. parapsilosis* and *C. tropicalis* strains (unexpectedly, *C. tropicalis* produces a cold-active enzyme while *P. antarctica* produces a thermostable one). The activation energy of the reactions catalysed by the two lipase/acyltransferases CpLIP2 and CtroL4 was on average 60 % lower than that of the CaLA and AflaL0 lipases (70 % lower when considering the alcoholysis reaction) and was associated with a high specific activity, demonstrating the high catalytic efficiency of these enzymes.

In addition to their peculiar ability to catalyse acyltransfer reactions in aqueous medium and other properties as previously reported (Briand et al. 1994; 1995a, b, c; Vaysse 1997; Neugnot et al. 2002; Osorio et al. 2009a; b; Tecelão et al. 2010; Husson et al. 2011; Neang et al. 2013), the cold-active properties of lipases such as the lipase/acyltransferase CpLIP2 have thus a potential interest for the reduction of the energy cost of the processes by avoiding heating and/or for industrial applications requiring cold to mild process conditions, such as for the synthesis or lipophilization of thermosensitive or highly reactive lipids derivatives. The lipases/acyltransferases studied here could be used in their free or immobilized form, in aqueous media containing small amounts of a nucleophile acceptor (e.g. alcohol) and directly emulsified with lipid substrates, without the use of organic solvent or control of a_w. The other advantage of such a process is that the recovery of the lipids products can be achieved by simple lipid/water separation and that the enzyme can be easily recycled, even in its free form by direct reusing of the water phase. We show here that the subgroup of the *C. albicans* lipase-like family in the CaLA superfamily of the Lipase Engineering Database (Widmann et al. 2010) comprises cold-adapted enzymes with exceptional acyltransferase properties in biphasic aqueous media. Common structural features of this group of enzymes might thus be favourable not only to the cold-activity but also to the acyltransfer ability. Further studies on the structure and properties of this group of exceptional enzymes are currently conducted in order to better understand their structure-function relationships.

Acknowledgments Authors thank the French Embassy in Cambodia and Montpellier SupAgro for their financial support.

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