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Mapping substrate selectivity of lipases from thermophilic fungi

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Abstract

New methods were adapted to screen, fast and easily, the lipase specificity (topo- or enantio-selectivity) on crude extracellular extracts from thermophilic fungi. Substrate acyl chain length specificity was tested using *p*-nitrophenyl esters and vinyl esters by the detection of released *p*-nitrophenolate anions in the first case and protonation of *p*-nitrophenolate anions (color diminution) in the second case. Enantioselectivity was tested using either the direct reaction rates on individual enantiomers of glycidyl butyrate or on competition between these enantiomers and resorufin esters (-butyrate or -acetate). Among a library of 44 thermophilic fungi, 10 strains were pre-selected (based on their capabilities to produce constitutively extracellular lipases) for further lipase specificity studies. The above methods were applied to lipases from these pre-selected fungi and also to other several lipases preparations from bacterial, fungal and mammalian origin. Remarkably, the method on competition allowed the accurate determination of the enantiomeric ratio (*E*), since experimental data fitted correctly with the *E* determined by classical chemical methods. Consequently, these methods can be applicable for screening selectivity in a high number of lipases or esterases from wild isolates or variants generated by directed evolution, using directly in the test, the substrate (i.e. esters) that will be worked out in a given process.

Keywords: Selectivity; High-throughput screening; Fungal lipases; Enantioselectivity

1. Introduction

Lipases have a great value as catalyst in organic synthesis. Chemists exploit their proprieties, such as high chemo, regio and enantioselectivity, on a wide range of reactions using natural and synthetic substrates. Many examples of their applications in biotechnology have been well reviewed recently [1,2]. However, by looking carefully, it appears that, in many cases, activity and selectivity of lipases for a given reaction are not completely satisfactory and a process could be improved by "tailoring" a more suitable enzyme.

The main bottle neck is the lack of screening procedures really adapted to the process that will be developed. By now, lipase producers (wild or recombinant strains) are selected on general criteria mainly based on the activity on olive oil or on the production level. However, it would be more convenient a lipase selection for a given reaction, based on the desired specificity. This remark is particularly important when lipases enantioselectivity is exploited for the preparation of high pure optical compounds, used as building blocks in drug synthesis.

There are two main sources of lipase biodiversity. First, different methods of protein engineering are now available to modify properties of existing native lipases by mutagenesis techniques (e.g. DNA shuffling, directed evolution). These methods generate thousands of mutants that must be screened for the desired properties on an industrial process. Second, nature biodiversity is still largely under-explored and it remains an important reservoir of new enzymes with the desired selectivities [3]. On the other hand, there is a lot of scope to search for new thermostables lipases [4]. Thermophilic fungi are potential sources of such lipases but they have remained largely under-explored compared to other thermophilic species belonging to bacteria and archaea domains. One possible explanation for this situation is that fungal thermophily is significantly lower (maximal growth temperatures are around 60 °C for fungi compared to 113 °C for archaea). It is worth noting that this fact is not a true disadvantage since moderate thermophilic species are good sources of enzymes possessing high catalytic activities at the temperature

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range usual for industrial processes (30–60 °C), with increased thermal stability compared to enzymes from mesophiles [5,6].

Actually, screening for enantioselectivity demands running a reaction for each lipase and measuring the ratio of enantiomers by HPLC, GC or NMR. Lipase acyl chain length specificity is classically determined by the pH stat method, using triglycerides as substrates. Unfortunately, these methods are so slow and tedious that they cannot be applied for screening of large amount of samples. Other spectrophotometrical methods are applied to estimate lipases and esterases enantioselectivity [7,8] and substrate acyl chain length specificity by using synthetic esters, which liberate a chromophore compound [3,9]. Such methods are useful as a first approach, but they can be misleading since they are not real substrates.

The main goal of this work was to develop a fast screening method for assaying lipases selectivity, applied to numerous enzymatic samples obtained from either wild or mutant isolates. In order to determine the true enantioselectivity on a lipases library obtained from 44 thermophilic fungi, we have adapted the Quick *E* method described by Somers and Kazlauskas [10]. Furthermore, the acyl chain length specificity on several non-chromogenic substrates (vinyl esters) was estimated by using *p*-nitrophenol (as a pH indicator) to detect hydrolysis as described by Janes et al. [11]. Finally, the enantioselectivity of our lipase library was compared to several lipases from different origins, by using the modified Quick *E* method and glycidyl butyrate as substrate and also compared to that *E* obtained by classical methods.

2. Materials and methods

2.1. Reagents and media

Vinyl esters (-acetate, -propionate, -butyrate, -laurate), *p*-nitrophenyl esters (-acetate, -propionate, -butyrate, -hexanoate, -octanoate, -decanoate, -laurate, -myristate and -palmitate), resorufin esters (-acetate and -butyrate), (*R*)-glycidyl butyrate, (*S*)-glycidyl butyrate, polyvinyl alcohol (PVA) 30,000–70,000 wt., Tween 80, Tris–HCl, BES, bovine serum albumin (BSA), corn steep liquor and potato dextrose agar (PDA) were purchased from Sigma–Aldrich, Fluka Chimie (St. Quentin-Fallavier, France). Vinyl esters (-hexanoate, -octanoate, -decanoate, -myristate and -palmitate) were from Tokio chemical industry Co. Europe (2070 Zwijndrecht, Belgium). Peptone was from United States biochemical Co. (44128 Cleveland, OH, USA). Enzymes from other organisms were kindly donated by Dr. R. Verger (Enzymology at Interfaces and Physiology of Lipolysis, CNRS, Marseille, France). All other chemicals and solvents were of reagent or better quality and were obtained from French suppliers.

2.2. Fungal strains conservation

The 44 thermophilic fungi recently isolated at the Bioprocess laboratory, were kept on potato dextrose agar (PDA) slants at 4 °C and stored on the culture collection of the Universidad de Guadalajara (Mexico). A preliminary identification of the isolated fungi, based on morphological observations, revealed that these strains could be identified as *Rhizopus* sp. (34 strains) and *Rhizomucor* sp. (10 strains). An inoculum was prepared by culturing each fungal strain in 250 ml Erlenmeyer flasks containing 50 ml of PDA at 30 °C for a week. Spores were harvested with 50 ml of a Tween 80 solution (0.01%).

2.3. Lipases production

Lipase production was performed in 250 ml Erlenmeyer flasks containing each 40 ml of the following culture medium (%, w/v): 4, corn steep liquor; 1, peptone; 1.4, KH_2PO_4; 0.24, K_2HPO_4; 0.04, $MgSO_4$ [12]. Initial pH of culture medium was adjusted to 6.4. The Erlenmeyer flasks were autoclaved at 121 °C for 15 min, inoculated with a spores' suspension to reach a final concentration of 5×10^5 spores/ml of culture medium and incubated at 40 °C and 170 rpm in a rotary incubator (INFORS AG CH 4103 Bottingen, Switzerland). For each fungal strain, the extracellular lipase activity was monitored each 4 h. When optimal activities were reached, the enzymatic broths were recovered and used for further studies.

2.4. Lipases assay

Lipase activity was measured by two methods: on emulsified *p*-nitrophenyl palmitate and olive oil according to Kordel et al. [9] with some modifications and to Kwon and Rhee [13], respectively. One volume of a 10 mM substrate solution in 2-propanol was mixed with nine volumes of 100 mM Tris–HCl (pH 8) containing PVA 0.25% (w/v). Then, this mixture was pre-warmed at 40 °C in a water bath and immediately distributed (1 ml) in 1.5 ml cells. The reaction was started by adding 0.5 ml of enzyme solution at an appropriate dilution in 10 mM Tris–HCl (pH 8). The increase in absorbance at 410 nm, against a blank without enzyme, was continuously monitored for 2–5 min using a UV–vis spectrophotometer (Shimadzu UV-160A, Shimadzu Corporation, Kyoto, Japan). Reaction rate was calculated from the slope of the absorbance curve versus time by using a molar extinction coefficient of 12,750 $cm^{-1}\,M^{-1}$ for *p*-nitrophenol. One enzyme unit was defined as the amount of protein releasing 1 μmole of *p*-nitrophenol per minute at the above conditions. Alternatively lipase activity was measured, using a colorimetric method based on the formation of copper soaps of free fatty acids. An emulsion was prepared by mixing in an Ultra-Turrax homogenizer (19,000 rpm, 10 min, 4 °C), 20 g of olive oil and 100 ml of a 0.1 M Tris–HCl buffer (pH 8 at 40 °C), containing 0.25 % PVA and 10 mM $CaCl_2$. Reaction was started by the addition of 50 μl of enzyme extract to 5 ml of emulsion and incubated for 15 min at 40 °C, stopping with 1 ml of 6 M HCl. Tubes were then heated in a boiling water bath for 5 min and the released fatty acids were extracted by vortex with 5 ml of isooctane for 1.5 min. This mixture was centrifuged ($5000 \times g$, 15 min) and 0.5 ml of a copper acetate–pyridine solution (50 g/l, pH 6.1 adjusted with pyridine) was mixed with 2.5 ml of recovered organic phase, by vortexing for 1.5 min. Absorbance was measured at 715 nm in a Shimatzu UV1205 spectrophotometer. A blank was prepared running a parallel assay without enzyme.

All assays were independently done in triplicate. A calibration curve was drawn against oleic acid using a range of concentrations from 0 to 10 mM. One unit of activity was defined as the amount of enzyme, which release 1 μmole of fatty acid per minute in the assay conditions.

3. Protein determination

Protein concentration was routinely determined using the Bradford [14] microassay procedure with Biorad dye reagent. BSA was used as the reference protein.

3.1. Lipases library

Fungal fermentations were carried out as described above, and the liquid cultures were filtered using paper Whatman no. 1. The culture broth filtrates containing lipase activity were distributed in aliquots (1.5 ml) and stored at −20 °C. Prior to the specificity screening studies, these aliquots were unfrozen and diluted 10-fold in a BES 2.5 mM buffer (pH 7.2).

4. Determination of lipases acyl chain length specificity

4.1. On p-nitrophenyl esters

The acyl chain length specificity was measured by using different *p*-nitrophenyl esters. One volume of the substrate (10 mM) dissolved in 2-propanol was mixed with nine volumes of 100 mM Tris–HCl (pH 8). When long acyl chain *p*-nitrophenyl esters (C-14 to C-18) were used, PVA 0.25% (w/v) was also incorporated in this buffer to prepare the substrate emulsions. Subsequently, 50 μl of each enzyme solution, at an appropriate dilution in 10 mM Tris–HCl (pH 8), were placed to each microplate well (96 wells); then, 100 μL of the different substrate solutions were added quickly using an eight channel pipette and the plate was placed on the microplate reader (Power wave X Select, BioTek Instruments, Inc.) and shaken for 5 s after each reading. The increase in absorbance at 410 nm was monitored at 25 °C every 25 s against a blank without enzyme. Data were collected in triplicate for 20 min. Reaction rate was calculated from the slope of the absorbance curve versus time by using a molar extinction coefficient of 8300 $cm^{-1}\,M^{-1}$ for *p*-nitrophenol (determined at the assay conditions). One enzyme unit was defined as the amount of protein liberating 1 μmole of *p*-nitrophenol per minute at the above conditions.

4.2. On vinyl esters

The acyl chain length specificity was measured by using different vinyl esters. Substrate emulsions were prepared as following: one volume of the substrate (50 mM) dissolved in acetonitrile, also containing 5 mM of *p*-nitrophenol (a pH indicator), was mixed with nine volumes of 2.5 mM BES (pH 7.2) containing 0.25% PVA (w/v). Then, 20 μl of each enzyme solution, at an appropriate dilution in BES 2.5 mM (pH 7.2), was placed in each microplate well and 100 μl of substrate emulsions was quickly added using an eight-channel pipette. Subsequently, the plate was positioned in the microplate reader and shaken for 5 s after each reading. The decrease in absorbance at 410 nm was recorded every 25 s at 25 °C. Blanks without enzyme were carried out for each substrate and data were collected in triplicate for 20 min. The reaction rates were calculated by means of the Eq. (1)

$$\text{rate}\,(\mu\text{mol/min}) = U = \frac{dA_{410}/dt}{\Delta\varepsilon_{410}\times l}\times\left(\frac{[\text{buffer}]}{[\text{indicator}]}+1\right)\times V\times 10^{6} \quad (1)$$

where dA_{410}/dt is the absorbance decrease at 410 nm/min determined by linear regression of absorbance versus time data, $\Delta\varepsilon_{410}$ is the difference in extinction coefficients for the protonated and unprotonated structures of the pH indicator (17,500 $M^{-1}\,cm^{-1}$), l is the path length (0.35 cm for a 120 μl reaction volume; in microplates) and V is the reaction volume in liters.

5. Determination of lipases enantioselectivity

Substrate solutions were prepared by mixing separately 500 μl of each glycidyl butyrate enantiomer (100 mM, dissolved in acetonitrile, containing 10 mM of *p*-nitrophenol as pH indicator), 500 μl of resorufin butyrate or resorufin acetate as reference compound (1 mM, dissolved in acetonitrile) and 9 ml of 2.5 mM BES (pH 7.2). Reactions were performed as described in the vinyl esters test, using the respective substrate solutions and monitoring simultaneously the decrease in absorbance at 410 nm for *p*-nitrophenol and the increase in absorbance at 570 nm for resorufin. Blanks without enzyme were carried out for each substrate and data were collected in triplicate for 20 min. Reaction rates for each enantiomer and reference compounds were calculated by means of the Eqs. (2) and (3), respectively, using the initial linear parts on the curves absorbance versus time.

$$\text{rate}_{\text{sub}}\,(\mu\text{mol/min}) = U_{\text{sub}} = \left[\frac{dA_{410}/dt}{\Delta\varepsilon_{410}\times l}\times\left(\frac{[\text{buffer}]}{[\text{indicator}]}+1\right)\times V\times 10^{6}\right] - 1.1[\text{rate}_{\text{ref}}] \quad (2)$$

$$\text{rate}_{\text{ref}}\,(\mu\text{mol/min}) = U_{\text{ref}} = \frac{dA_{570}/dt}{\Delta\varepsilon_{570}\times l}\times V\times 10^{6} \quad (3)$$

where dA_{570}/dt is the absorbance increase at 570 nm/min, $\Delta\varepsilon_{570}$ is the difference in extinction coefficients for the protonated and unprotonated resorufin forms (15,100 $M^{-1}\,cm^{-1}$). Enantioselectivity was calculated from the hydrolysis rate for each enantiomer ($U_{(S)\text{-sub}}$ and $U_{(R)\text{-sub}}$) and its correspondent reference compound (U_{ref}) with the Eq. (4).

$$\text{Quick } E = \frac{U_{(S)\text{-sub}}[\text{reference}]/U_{\text{ref}}[(S)\text{-sub}]}{U_{(R)\text{-sub}}[\text{reference}]/U_{\text{ref}}[(R)\text{-sub}]} \quad (4)$$

For the enantioselectivity estimation (without competition) and specific activity determination, 500 μl of acetonitrile was added instead of the reference compound in the above test. The decrease in absorbance at 410 nm was monitored for each enantiomer, and reaction rates were calculated by means of the Eq.

Table 1
Maximal lipase activities of thermophilic fungi and total protein concentrations in crude broth extracts

Fungal strains	Lipase activity		Ratio (olive oil/*p*-NPP)	Total protein (mg/ml)	Time (h)[a]
	p-NPP (U/ml)	Olive oil (U/ml)			
8a	**5.3**	**54**	10.2	0.53	16
9a	0.99	9.5	9.6	0.51	15
13a	**3.6**	**42**	11.7	0.31	24
13b	1.9	21	11.1	0.49	18
19	2.6	20.2	7.8	0.51	21
23a	0.207	4.4	21.3	0.40	20
25c	1.9	20.7	10.9	0.37	15
42c	0.128	<0.100	<0.8	0.59	20
43a	2.8	10.14	3.6	0.38	16
56c	3.1	9.7	3.1	0.66	22

Values are the average of three independent assays with S.D. less than 5%.
[a] Time is the fungal culture time where a maximal lipase activity was reached.

(1), using the initial linear parts on the absorbance versus time curves.

5.1. Endpoint method

A suitable amount of lipase was added to 10 ml of 40 mM racemic mixture of glycidyl butyrate dissolved in 50 mM Tris–HCl buffer (pH 7), containing 10% (v/v) of acetonitrile. Reactions were maintained under magnetic stirring at 200 rpm and 25 °C. At different reaction times (0.25, 0.5, 1, 2, 4, 8, 16 and 32 h), residual substrates and products were extracted by mixing 0.5 ml of reaction aqueous solution and 0.25 ml of ethyl acetate. The enantiomeric composition in the organic phase was determined by chiral gas chromatography (CGC) using a Lipodex E chiral column (25 m × 0.25 mm). Chromatographic conditions were injector temperature, 250 °C; oven temperature, 65 °C; flame ionization detector temperature, 200 °C; gas carrier flow (helium), 1.5 ml/min. The retention times for the different enantiomers were; (*R*)-glycidol = 8.2 min; (*S*)-glycidol = 9 min; (*R*)-glycidyl butyrate = 29.6 min; (*S*)-glycidyl butyrate = 31.2 min.

The enantioselectivity ratio (*E*) was calculated by the Eq. (5) reported by Rakels et al. [15].

$$E = \frac{\ln[(1 - e.e_s)/(1 + e.e_s/e.e_p)]}{\ln[(1 + e.e_s)/(1 + e.e_s/e.e_p)]} \quad (5)$$

where $e.e_s$ and $e.e_p$ are, respectively, the enantiomeric excess of substrates and products.

6. Results and discussion

6.1. Lipase library

Thermophilic fungal strains from our collection were cultivated in liquid fermentations. Kinetics of growth and lipase production were followed until 30 h of culture, finding a correlation between both cultural parameters (data not shown). Among 44 strains tested, only 10 of them produced constitutively extracellular lipases. It is worth noting that lipase activity was detected at the early stage of growth (4 h of culture) for six strains (43a, 13b, 56c, 19, 25c and 8a), while for the other four strains (13a, 9a, 42c and 23a), it was detected after 10 h of lag phase. For these 10 strains, maximal lipase activities were reached within 15–24 h before declining and they are reported in Table 1. These lipases were able to hydrolyze *p*-NPP and olive oil. This last result confirms that true lipase activities were present in all the crude extracts. In Table 1, it is also observed that 8a and 13a strains showed the highest lipase activities on both substrates tested (5.3 and 3.6 U/ml on *p*-NPP and 54 and 42 U/ml on olive oil, respectively). Furthermore, lipase activity on olive oil was generally higher than on *p*-NPP (4–20 times). This observation is in agreement with that reported by Beisson et al. [16].

7. Determination of lipases acyl chain length specificity

7.1. Specificity on p-nitrophenyl esters

The colorimetric assay using *p*-nitrophenyl esters of variable chain-length fatty acids is a very simple and sensitive spectrophotometrical technique that is largely used to study lipases specificity [5,17–19]. In order to screen chain length specificity from the 10 pre-selected fungal lipases, various *p*-nitrophenyl esters were used. As shown in Table 2, all lipases tested hydrolyzed preferentially medium length *p*-nitrophenyl esters (C6–C8), with relatively low activity (less than 10%) on short chain fatty acids (C2–C4), particularly for those lipases from 23a and 42c strains (less than 1%). Notably, lipases from 8a, 43a and 56c strains showed a high activity on long chain *p*-nitrophenyl esters (C16–C18), compared to that activity observed (>50%) for medium length *p*-nitrophenyl esters (C6–C8).

The observed preference for medium chain length fatty acids can be explained by the following two annotations: (1) the thermophilic fungal strains employed in this study were isolated from coconut copra which contains mainly triglycerides of medium chain length fatty acids (C6–C12) [20,21]. (2) These strains were classified into *Rhizopus* and *Rhizomucor* genera,

Table 2
Acyl chain length specificity of thermophilic fungal lipases on different *p*-nitrophenyl esters

Acyl chain length	Lipase activity (U/ml)									
	8a[a]	9a[a]	13a[a]	13b[a]	19[a]	23a[a]	25c[a]	42c[a]	43a[a]	56c[a]
C2	0.82	0.08	0.33	0.19	0.41	<0.003	0.22	<0.003	0.38	0.39
C3	1.15	0.16	0.88	0.39	0.62	0.006	0.43	0.008	0.71	0.62
C4	2.95	0.45	2.22	1.02	1.70	0.03	1.30	0.01	1.94	1.71
C6	**8.24**	1.98	9.08	3.28	5.40	**0.77**	**4.75**	**0.41**	3.38	3.37
C8	7.79	**2.08**	**9.78**	**3.47**	**5.68**	0.67	4.68	**0.41**	**4.25**	**5.19**
C12	4.92	1.47	6.19	2.16	4.05	0.50	2.95	0.39	3.90	3.97
C14	5.29	0.61	2.93	1.54	2.56	0.19	1.66	0.07	2.29	2.65
C16	4.43	0.82	3.15	1.58	2.18	0.17	1.58	0.11	2.41	2.62
C18	4.51	0.63	2.48	1.32	1.97	0.15	0.76	0.01	2.01	2.54

Values are the average of three independent assays with S.D. less than 5%.
[a] Strains.

which are recognized to produce lipases hydrolyzing preferentially medium chain length (C6–C12) fatty acid esters [12,22].

The determination of fatty acid specificity using *p*-nitrophenyl esters is easy and convenient; however, there are two main limitations of this approach: (1) this assay cannot be used at pH values lower than the *p*-nitrophenol pK_a (7.2), due to its loss of absorbance. (2) The existence of a *p*-nitrophenyl group (a hydrophobic and voluminous group) in the substrate may affect enzyme specificity. In order to avoid these limitations and to propose a more applicable test, vinyl esters were used as substrates.

7.2. Specificity on vinyl esters

Vinyl esters of different acyl chain length (C2–C16) were used as substrates and their hydrolysis was monitored by the release of protons using *p*-nitrophenol as a pH indicator, according to Somers et al. [10] (Fig. 1a). Soluble vinyl esters, containing acyl chains ranging between C2–C4, were found to be very poor substrates for all the 10 fungal lipase preparations (Table 3). This result agrees with that obtained using *p*-nitrophenyl esters as substrates (Table 2) and indicated that the main enzymatic activity in the tested broths is a lipase activity

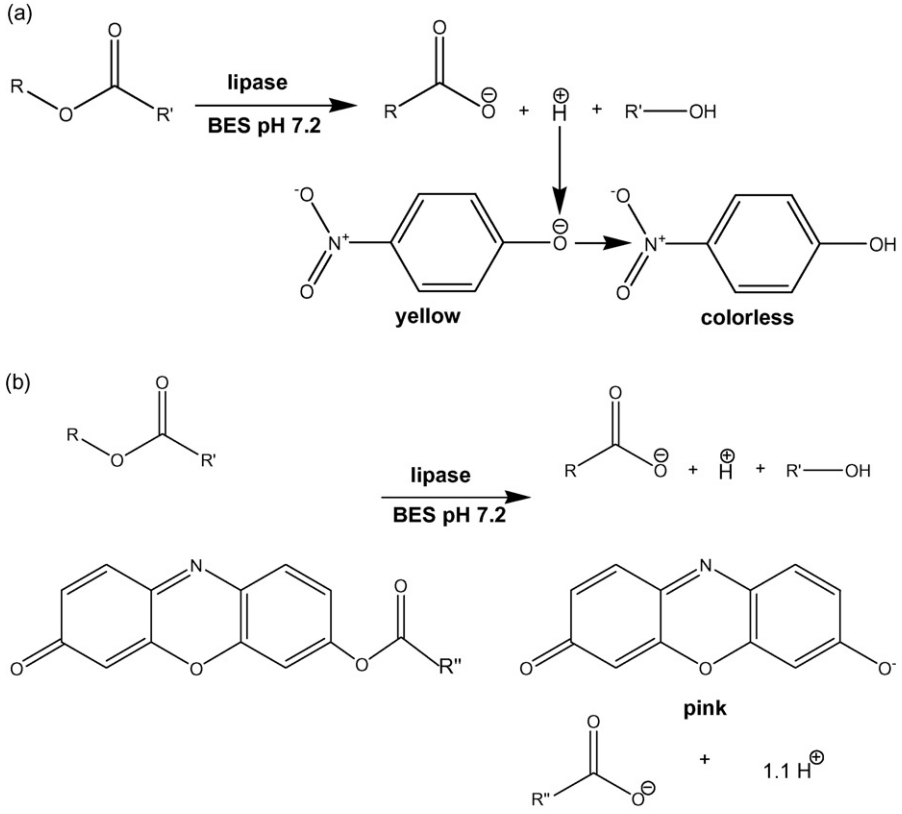

Fig. 1. Measuring selectivity of lipases. (a) Estimated selectivities compared the initial rates of hydrolysis of different esters measured spectrophotometrically using *p*-nitrophenol as pH indicator. The yellow coloration of *p*-nitrophenolate monitored at 410 nm diminishes as hydrolysis proceeds. (b) Competitive experiment. Resorufin butyrate or resorufin acetate were used as competitive substrates, their hydrolysis generate pink coloration (resorufin anion) monitored at 570 nm.

rather than an esterase one. Furthermore, lipase activity obtained on vinyl butyrate was also confirmed by using the pH stat method at the same conditions above employed (vinyl butyrate concentration 5 mM, pH 7.2, 25 °C and without agitation). Analogous activities were found by using both methods (data not shown).

Generally, the enzymatic fungal extracts showed the highest lipase activity with vinyl caprylate (76–100% of the maximum activity). Nevertheless, 8a and 42c strains preferentially hydrolyzed vinyl laurate (C12), while 13a strain had the highest lipase activities as much in vinyl caprylate (C8) as in vinyl myristate (C14). Both lipase activities values (∼5.7 U/ml) were twice bigger than values obtained for medium chain length substrates.

Surprisingly, for all fungal lipases, nearly no activity was detected under experimental conditions using vinyl palmitate (C16) as substrate. Since *p*-nitrophenyl palmitate was significantly hydrolyzed by all the fungal lipases (Table 2), it would be predicted that lipases would hydrolyze vinyl palmitate. In this sense, it should be mentioned that technical difficulties were encountered by using vinyl palmitate. Because this compound is solid and water insoluble, it was dissolved in acetonitrile at 60 °C and then emulsified in a buffer containing PVA. It is possible that some precipitation occur during the assay of lipase activity especially in non-agitated microplates resulting in not completely reliable results. Thus we recommend not to use vinyl palmitate under these conditions.

When using vinyl esters the fatty acid pK_a is an important factor since it varies with chain length [23]. Contrarily to the *p*-nitrophenyl esters tests where the release of a chromophore group is measured, for the vinyl ester assay, the protons released are measured using *p*-nitrophenol as indicator. Thus the degree of acid dissociation is crucial. For example, pKa for butyric and palmitic acids are, respectively, ∼5 and ∼9 [23] resulting in incomplete deprotonation at neutral pH and thus underestimation of lipase activity. We had also observed non-linear kinetics (data not shown) for long chain fatty acid esters.

7.3. Competition experiments

Fatty acid specificity should be more accurately determined by using competition between substrates as previously reported in our laboratory [24] and by others [25]. This approach was assayed by introducing resorufin butyrate as a competitor, in order to get the true specificity as described by Somers et al. (Quick *S*) [10]. However, it is worth noting that the major difficulty for the determination of lipases specificity is the heterogeneity of the reaction medium, since it is based on Michaelis–Menten kinetics, i.e. with soluble substrates [26–28]. In this work, we hypothesized that the first step on the reaction, the lipase adsorption on the oil–water interface, was faster than the ester hydrolysis and that the Michaelis–Menten theory applies at the interface for two competing substrates.

With this in mind, competition experiments between vinyl esters and resorufin butyrate were performed, finding contradictory results. Compared to a test using only resorufin butyrate, upon competition, the hydrolysis rate of resorufin butyrate increased more than five-fold (instead of decreasing) when long acyl chain vinyl esters were added in the reaction mixture. A feasible explanation to this phenomenon is the incorporation of resorufin butyrate into long vinyl esters micelles, which might have increased its hydrolysis rate. A second explanation is a possible change in the interface quality, which may affect the enzyme activity. Several attempts to overcome these limitations were unsuccessful; therefore, we conclude that the determination of the lipases true selectivity, using the above competition experiments is not possible.

8. Determination of lipases enantioselectivity

In the present tests, glycidyl butyrate was chosen as a model molecule, since chiral glycidols are used as intermediates in the synthesis of many pharmaceuticals compounds.

8.1. Estimation of lipases enantioselectivity without competition

The initial rate of hydrolysis for each glycidyl butyrate enantiomer was measured separately by monitoring the release of protons using *p*-nitrophenol as a pH indicator (Fig. 1a), as described by Janes et al. [11] and as done previously for vinyl esters. Estimation of lipases enantioselectivity was then cal-

Table 3
Acyl chain length specificity of thermophilic fungal lipases on different vinyl esters

Acyl chain length	Lipase activity (U/ml)									
	8a[a]	9a[a]	13a[a]	13b[a]	19[a]	23a[a]	25c[a]	42c[a]	43a[a]	56c[a]
C2	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003
C3	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003
C4	<0.003	<0.003	<0.003	<0.003	0.105	<0.003	<0.003	<0.003	<0.003	<0.003
C6	3.74	1.16	1.15	0.15	1.48	0.1	1.02	1.37	1.48	1.21
C8	8.46	**4.96**	5.59	**1.78**	**4.87**	**0.88**	**5.7**	5.89	**6.80**	**4.91**
C10	4.33	2.17	2.23	0.62	2.32	0.36	2.44	2.95	3.08	2.42
C12	**10.22**	2.54	2.77	0.15	2.69	0.05	3.03	**7.73**	3.96	3.38
C14	1.97	3.91	**5.78**	0.68	1.19	0.58	4.64	1.41	1.41	1.33
C16	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003

Values are the average of three independent assays with S.D. less than 5%.
[a] Strains.

Table 4
Specific activities and estimated enantioselectivities (without competition) of lipases from fungi (Group I and IIA), bacteria (Group IIB) and mammals (Groupe IIC) determined by using glycidyl butyrate enantiomers

Lipases	Specific activity (U/mg)		Ratio ((*S*)/(*R*))
	(*S*)	(*R*)	
Group I (fungi)			
8a[a]	0.42 ± 0.016	0.14 ± 0.013	2.9
19[a]	0.33 ± 0.012	0.16 ± 0.006	2.0
56c[a]	0.42 ± 0.017	0.14 ± 0.004	2.9
13b[a]	0.41 ± 0.012	0.13 ± 0.012	3.1
43a[a]	0.65 ± 0.005	0.19 ± 0.05	3.3
25c[a]	0.37 ± 0.021	0.18 ± 0.02	2.1
42c[a]	0.19 ± 0.008	0.14 ± 0.0011	1.4
23a[a]	0.28 ± 0.009	0.12 ± 0.043	2.3
9a[a]	0.15 ± 0.021	0.12 ± 0.005	1.3
13a[a]	0.51 ± 0.01	0.13 ± 0.002	**3.9**
13a[b]	23.8 ± 0.94	5.7 ± 0.03	**4.1**
Group II (A) (fungi)			
Rhizopus oryzae (ROL)[c]	0.78 ± 0.02	0.14 ± 0.005	5.4
Rhizopus delemar (RDL)[b]	162.7 ± 5.25	32.6 ± 1.9	5.0
Mucor javanicus (MJL)[c]	0.1 ± 0.004	0.014 ± 0.0085	6.9
Humicola lanuginose (HLL)[b]	23.5 ± 1.3	7.4 ± 0.38	3.2
Aspergillus niger (ANL)[c]	<0.001	<0.001	∼1.0
Penicillium camembertii (PcmL)[c]	0.092 ± 0.06	0.866 ± 0.06	1.1
Penicillium simplicissimum (PsL)[b]	0.53 ± 0.03	0.55 ± 0.045	1.0
Candida rugosa (CrL)[c]	0.043 ± 0.0015	0.036 ± 0.0014	1.2
Candida antarctica A (CaAL)[b]	23 ± 0.52	15 ± 0.23	1.5
Candida antarctica B (CaBL)[b]	21.1 ± 1.34	85.7 ± 1.5	**0.25**
Fusarium solani (FsL)[b]	16.9 ± 0.34	14.35 ± 0.7	1.2
Group II (B) (bacteria)			
Pseudomonas cepacia (PcL)[c]	0.65 ± 0.024	0.135 ± 0.0027	**4.9**
Pseudomonas glumae (PgL)[b]	25.3 ± 0.66	19.23 ± 0.7	1.3
Pseudomonas fluorescens (PfL)[b]	1.77 ± 0.1	0.48 ± 0.01	3.7
Bacillus subtilis (BsLA)[b]	0.41 ± 0.024	0.28 ± 0.016	1.5
Group II (C) (mammals)			
Human pancreatic lipase (HPL)[b]	23.44 ± 0.23	2.82 ± 0.26	**8.3**
Dog pancreatic lipase (DPL)[b]	6.75 ± 0.3	1 ± 0.066	6.7
Pig pancreatic lipase (PPL)[b]	16.1 ± 0.3	1.92 ± 0.03	**8.4**
Horse pancreatic lipase (HoPL)[b]	0.15 ± 0.0014	0.024 ± 0.002	6.1
Human pancreatic lipase type 2 (HPLRP2)[b]	2.53 ± 0.009	0.69 ± 0.075	3.6
Guinea pig pancreatic lipase type 2 (GPLRP2)[b]	15.36 ± 0.8	5.78 ± 0.36	2.6
Rat pancreatic lipase type 2 (RPLRP2)[b]	0.52 ± 0.03	0.092 ± 0.01	5.7
Lipoprotein lipase (LPL)[b]	2.56 ± 0.03	2.61 ± 0.02	1.0

Values are the average of three independent assays.
[a] Broth extract.
[b] Purified lipases.
[c] Commercial lipases.

culated from the ratio of initial rates of (*S*)- and (*R*)-glycidyl butyrate enantiomers. By applying this test, enantioselectivity of our fungal lipase library (Table 4, group I) were compared to several lipases (Table 4, group II), kindly donated by Dr. Verger (Lab. of Enzymology at Interfaces and Physiology of Lipolysis, CNRS, Marseille France) from fungal (group IIA), bacterial (group IIB) and mammalian (group IIC) origin. The perspective of this study was to discern whether the enantioselectivity screening test could be applicable among lipases from different origins.

Most of the lipases tested hydrolyzed preferentially (*S*)-glycidyl butyrate with (*S*)/(*R*) ratios ranging from 1.0 to 8.4 (Table 4). A remarkable exception was *Candida antartica* lipase B (CaB), which hydrolyzed preferentially the (*R*)-glycidyl butyrate with a (*R*)/(*S*) ratio of 4. This lipase also showed high specific activities for both enantiomers (21 and 86 U/mg, respectively, for *S* and *R* enantiomers). Among the 10 crude enzymatic extracts from group I, lipase from 43a strain displayed the highest specific activity towards (*S*)-glycidyl butyrate (0.65 U/mg). This activity value was comparable to commercial lipases from *Rh. oryzae* and *Pseudomonas cepacia* (Table 4). Among the purified lipases, *Rh. delemar* lipase (RDL) had a very high specific activity towards the (*S*)-enantiomer (162.7 U/mg), and the purified lipase from 13a strain exhibited a specific activity similar than other fungal (HLL, CAA), bacterial (PgL) and mammalian (HPL) purified lipases in group II (23–25 U/mg).

Table 5
Enantioselectivity ratio (*E*) of lipases from fungi (Group I and IIA), bacteria (Group IIB) and mammals (Group IIC)

Lipase	*E*		Preferential enantiomer
	Resorufin acetate[a]	Resorufin butyrate[a]	
Group I (thermophilic fungi)			
8a[b]	2.5 ± 0.23	3.2 ± 0.38	(*S*)
19[b]	2.5 ± 0.78	2.8 ± 0.31	(*S*)
56c[b]	3.0 ± 0.80	2.5 ± 0.71	(*S*)
13b[b]	2.5 ± 0.11	1.4 ± 0.31	(*S*)
43a[b]	2.3 ± 0.07	1.6 ± 0.10	(*S*)
25c[b]	N.D.	1.1 ± 0.15	(*S*)
42c[b]	N.D.	1.1 ± 0.070	(*S*)
23a[b]	N.D.	1.1 ± 0.023	(*S*)
9a[b]	N.D.	1.1 ± 0.025	(*S*)
13a[b]	**5.5 ± 0.35**	N.D.	(*S*)
13a[c]	**5.8 ± 0.47**	2.1 ± 0.28	(*S*)
Group II (A) (commercial fungi)			
Rhizopus oryzae (ROL)[d]	3.7 ± 0.04	3.7 ± 0.22	(*S*)
Rhizopus delemar (RDL)[c]	3.7 ± 0.32	2.3 ± 0.1	(*S*)
Mucor javanicus (MJL)[d]	**5.4 ± 0.34**	7.1 ± 0.58	(*S*)
Humicola lanuginosa (HLL)[c]	3.4 ± 0.07	3.8 ± 0.01	(*S*)
Aspergillus niger (ANL)[d]	N.D.	1.4 ± 0.18	(*S*)
Penicillium camembertii (PcmL)[d]	1.3 ± 0.01	1.1 ± 0.025	(*S*)
Penicillium simplicissimum (PsL)[c]	N.D.	1.0 ± 0.065	(*S*)
Candida rugosa (CrL)[d]	2.5 ± 0.14	2.8 ± 0.15	(*S*)
Candida antarctica A (CaAL)[c]	1.3 ± 0.06	1.0 ± 0.06	(*S*)
Candida antarctica B (CaBL)[c]	**3.8 ± 0.38**	3.5 ± 0.16	(*R*)
Fusarium solani (FsL)[c]	1.3 ± 0.06	1.2 ± 0.01	(*S*)
Group II (B) (bacteria)			
Pseudomonas cepacia (PcL)[d]	3.6 ± 0.50	**3.7 ± 0.05**	(*S*)
Pseudomonas glumae (PgL)[c]	1.4 ± 0.04	1.4 ± 0.1	(*S*)
Pseudomonas fluorescens (PfL)[c]	2.6 ± 0.13	3.1 ± 0.015	(*S*)
Bacillus subtilis (BsLA)[c]	N.D.	N.D.	N.D.
Group II (C) (mammals)			
Human pancreatic lipase (HPL)[c]	5.5 ± 0.92	**12.4 ± 1.63**	(*S*)
Dog pancreatic lipase (DPL)[c]	N.D.	4.7 ± 0.55	(*S*)
Pig pancreatic lipase (PPL)[c]	11 ± 0.17	2.2 ± 0.27	(*S*)
Horse pancreatic lipase (HoPL)[c]	4.2 ± 0.22	2.2 ± 0.2	(*S*)
Human pancreatic lipase type 2 (HPLRP2)[c]	3.7 ± 0.2	2.3 ± 0.4	(*S*)
Guinea pig pancreatic lipase type 2 (GPLRP2)[c]	2.4 ± 0.22	2.4 ± 0.17	(*S*)
Rat pancreatic lipase type 2 (RPLRP2)[c]	N.D.	5.9 ± 0.37	(*S*)
Lipoprotein lipase (LPL)[c]	1.0 ± 0.019	1.0 ± 0.038	(*S*)

(N.D.) Not determined because one of the substrates or reference compounds reacted too fast or too slow to be measured accurately. *E* values were obtained by the "Quick *E*" method using glycidyl butyrate enantiomers. Resorufin esters (acetate and butyrate) were used as reference compounds to introduce competition in the reaction. Values are the average of three independent assays.

[a] Reference compound.
[b] Broth extract.
[c] Purified lipase.
[d] Commercial lipase.

Among the 33 lipases hydrolyzing preferentially the (*S*)-glycidyl butyrate, 7 lipases showed very poor enantioselectivity with ratios lower than 1.5, whereas 16 lipases showed moderate enantioselectivity (1.5–4) and 10 lipases showed good enantioselectivity (4–8.4). These results confirm that this screening test might be applicable to determine the enantioselectivity of lipases from different origins. However, the selection of an ideal enzyme requires both, the highest enantioselectivity and specific activity. Therefore, pancreatic lipases like HPL and PPL as well as lipases from fungal origin like RDL, CaB and 13a (from our library) are good candidates for resolving a glycidyl butyrate racemic mixture.

It is important to point out that by measuring separately hydrolysis rate of each enantiomer does not take into account the competitive binding effect between substrates in an enzymatic active site, and measurements of true enantioselectivity require the competition between substrates [25].

8.2. Determination of true enantioselectivity by introducing competition

The true lipase enantioselectivity for glycidyl butyrate enantiomers was measured using a more accurate method (Quick *E*), described by Liu et al. [29]. The competition effect was intro-

duced by using either resorufin butyrate or resorufin acetate. These compounds were selected as a reference, because their hydrolysis generates a pink coloration (resorufin), which is ease to be monitored (Fig. 1b). In order to measure accurately a true enantioselectivity, it is mandatory that the reference compound (competitor) and the substrate react at a similar rate and they should be totally soluble.

As observed in Table 5, for the enantioselectivity with competition, the bulk of the enzymes tested hydrolyzed preferentially the (*S*)-glycidyl butyrate enantiomer. Exceptionally, CaB lipase hydrolyzed preferentially the (*R*)-enantiomer with an *E* value of approximately 4, in the presence of any of both resorufin esters (-acetate or -butyrate).

In the case of the bacterial lipases group, both competitors (resorufin esters) and substrates (glycidyl esters) were hydrolyzed at similar rates (Table 5). Extraordinarily, *Bacillus subtilis* lipase hydrolyzed extremely fast both competitors and it was therefore impossible to calculate the enantioselectivity ratio for this enzyme. For most fungal lipases, both competitors reacted too fast in comparison to the substrates. On the contrary, pancreatic lipases hydrolyzed slowly both competitors; however, the enantiomeric ratio was accurately calculated at least for one competitor (Table 5). These observations reveal the importance of selecting the right competitor for a given lipase to obtain a precise *E* value.

Interestingly, among all microbial lipases tested for enantioselectivity, 13a lipase from our fungal collection showed the best enantiomeric ratio for (*S*)-glycidyl butyrate enantiomer ($E = 6$). However, the highest enantiomeric ratios found in this work, were for pancreatic lipases HPL and PPL with *E* values of 12.4 and 11, respectively (Table 5). Surprisingly, Guinea pig pancreatic lipase 2 (GPLRP2) showed a lower *E* value (2.4) compared to the *E* calculated for HPL. GPLRP2 shows a very high structural homology with HPL, with the exception of the lid domain, which is constituted by five amino acid residues (a mini-lid) instead of 23 in HPL [30]. Thus, it appears that the HPL lid domain could be involved in the enantiomer recognition of (*S*)-glycidyl butyrate.

Finally, we also determined the enantioselectivity for the purified lipase isolated from 13a strain, by the classical endpoint method, in order to establish the precision of the Quick *E* measurements. The enantiomeric composition of products and substrates assayed by gas chromatography gave an *E* value of 7.5. This value was in good agreement with that determined by the Quick *E* method ($E = 6$). On the other hand, the *E* value calculated for the CaB lipase towards the *R*-glycidyl butyrate enantiomer (3.8), using the Quick *E* method was similar to that obtained by Palomo et al. [31] employing the same substrate and the endpoint method ($E = 4.6$). These two comparisons confirm the usefulness and accuracy of the Quick *E* method for the determination of the enantioselectivity of a large amount of enzymatic samples.

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