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Causes of Unreproducibility of *C. rugosa* Lipase-catalyzed Reactions in Slightly Hydrated Organic Media

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Abstract

Lipase activity, measured as hydrolysis of tributyrin is a valid assay to quantify the lipase activity of a lyophilized crude lipase in hydrolysis reactions but it is not useful to predict the catalytic activity in lipase-catalyzed reactions in organic media. Three factors control the catalytic activity in these media: i) relative proportion of isoenzymes; ii) amount of water in the lyophilized crude enzyme and iii) amount of lipase protein in the commercial powder. Thus we propose two simple reaction tests: i) heptyl oleate synthesis (specific of lipases), ii) enantioselective esterification of (**R**) or (**S**) 2-(3-benzoyl)phenyl propionic acid. This methodology is applied to different crude lipases of *Candida rugosa*, obtained in different fermenter conditions and shows the origin of the unreproducibility of the synthetic data.

Keywords: Lipase of *Candida rugosa*, lipase-catalyzed reactions, isoenzymes.

1. Introduction

Lipases (E.C. 3.1.1.3) comprise a group of enzymes which catalyze, *in vivo*, the hydrolysis of triacylglycerols. The development of technologies using lipases for the *in vitro* synthesis of chiral and/or novel compounds will result in their expansion into new areas and increase the number of industrial applications [1-3]. The use of these enzymes as common biocatalysts in organic synthesis laboratories and in the chemical industry is only slowly increasing despite mild reaction conditions and good enantioselectivities obtained in lipase-catalyzed reactions. One of the main reasons argued by organic chemists is the lack of reproducibility obtained in lipase-catalyzed reactions when commercial lipases from different batches of the same supplier are used, in spite of the same number of lipase units indicated by the supplier. These problems are very important for example when the lipase from the same microorganism gives different enantiopreference as reported in the case of *Rhizomucor miehei* lipase from Amano and from Novo Nordisk in the resolution of racemic 2-(3-benzoyl)phenyl propionic acid

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(ketoprofen) [4]. In the present paper we try to explain these observed differences and how they can be overcome by some simple reaction tests.

The imperfect yeast *Candida rugosa* (previously named *Candida cylindracea*) is a major source of commercial lipase (CRL) broadly employed by various laboratories and industrial applications [5,6] and classified as a non-specific lipase with respect to the position of the fatty acid chain released from the glycerol molecule. However, recently it has been recognised that CRL is indeed a heterogeneous mixture of different proteins since *C. rugosa* synthesises and secretes multiple lipase isoenzymes [7] differing in biochemical properties [6,8] and in substrate specificity [6,9].

Two isoenzymes have been isolated - isoenzymes A and B [8]. These isoforms have similar molecular weight, similar amino acid content (78%) but they differ in N-terminal sequences, hydrophobicity [9,10], specificity [6,10] and pI [8]. Very little is currently known of the regulation, organization and evolution of *C. rugosa* lipase genes and, generally speaking, molecular information about this yeast is still very sparse, possibly because other *Candida* species have called for more intensive investigation due to their pathogenicity and their impact on public health.

In the present paper we will show how, depending on the fermenter conditions and of the inducer used to grow the microorganism, the percentage of isoenzymes and the amount of water in the lyophilized powder, are different and so, the *in vitro* catalytic activity is different too.

2. Results

2.1.-Hydrolytic activity

Commercial suppliers sell the lipase of *Candida rugosa* with a clear specification of the number of hydrolytic units that may be easily evaluated. For example two lots of lyophilized lipase of *Candida rugosa* from Sigma Chemicals were tested in the hydrolysis of tributyrin (see experimental) obtaining:

batch 54HO260 (L 54)	133 U/mg dry powder
batch 85HO629 (L 85)	124 U/mg dry powder

with a relative variation of 9%, that is acceptable. Nevertheless the protein content of the crude powder varies in this case from 3 to 12%. If we want to use these biocatalysts in organic synthesis, three questions come to mind:

i) Would the most active crude enzyme be the most active biocatalyst using another hydrolysis assay?

ii) Would the enzymatic activity be affected by the fermenter conditions?

iii) Would the most active crude enzyme in the hydolysis-catalyzed reaction be the most active enzyme in synthesis reactions in organic media using unnatural substrates?

To answer these questions the yeast *Candida rugosa* was fermented in a minimum salt medium using different inducers as the only carbon source and different percentages of inducers [11]. These fermenter conditions were used to avoid the masking effect in enzyme biosynthesis due to a rich fermenter medium. The enzymatic activity of the supernatant broth in the hydrolysis of p-nitrophenylacetate (esterase activity) and of tributyrin (lipase

activity) was measured. The results obtained are shown in Table 1.

Table 1.- Hydrolitic activity of several crude lipases obtained using differents inducers as the carbon source.

Inducer	Percentage (%)	Esterase activity (U/ml)[a]	Lipase activity (U/ml)[a]
olive oil	2	99 ± 9	7.3 ±0.9
	5	111 ±15	5.6 ± 0.6
	10	157 ±15	5.4 ± 0.5
dodecanol	2	< 2	1.04 ±0.03
	5	89 ± 3	1.9 ± 0.2
	10	96 ± 6	1.7 ± 0.3
oleic acid	2	29 ± 11	2.49 ± 0.03
	25	31 ± 3	3.0 ± 0.4
	25	41 ± 4	3.3 ± 0.5
	5	102 ± 6	4.00 ± 0.03
	10	102 ± 33	2.31 ± 0.03
sunflower oil	2	74 ± 3	< 0.2
	5	62 ± 6	< 0.2
	10	84 ± 6	< 0.2

[a]: Units per ml of cell culture medium after cell removal.

From the results in Table 1, we note:

i) the lipase activity (hydrolysis of tributyrin) is very reproducible as an example, in the case of oleic acid (0.25%) as inducer: 3.3 and 3.0 U/ml (with an experimental deviation of 9% as in the data of the commercial powder commented above);

ii) when another hydrolysis reaction is used (i.e.: hydrolysis of p-nitrophenylacetate) the reproducibility decreases: 41 and 31 U/ml (variation of 25%);

iii) depending on the inducer and its percentage the enzymatic activity is different being a maximum with olive oil as inducer;

iv) the number of esterase and lipase units increase with the percentage of inducer but this is not general;

v) the variation in the number of units is greater in the esterase activity than in the lipase activity;

Therefore, we can conclude that the lipase activity using tributyrin hydrolysis as the reaction test, gives the most repetitive results. So, this magnitude is generally given by the suppliers to sell the crude lipase. Nevertheless the esterase activity (easy to measure by UV-visible spectroscopy) is not as repetitive as the lipase activity. These

values of enzymatic activity are strongly dependent on the inducer and its percentage as we can see in the case of olive oil and oleic acid (both at 0.5 or 1.0% inducer percentage). This is due to the presence of other enzymes (different lipases) in the crude powder as discussed later.

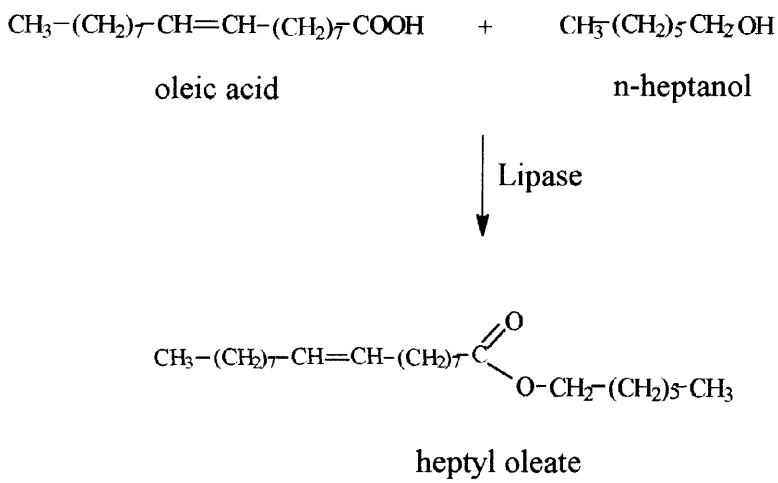
Therefore the fermenter conditions (nature and percentage of the inducer), give crude lipases with very different values in the hydrolytic activities (Table 1). This point is not generally described by the suppliers of enzymes for the Organic Chemistry laboratories.

From Table 1 we could suggest to the organic chemists that they must ask to the suppliers for: i) the fermenter conditions and ii) the enzymatic assay used by the supplier, which should be hydrolysis of tributyrin, as the repetitivity of the assay.

2.2-Synthetic reactions in organic media

Two main questions remain unanswered from the hydrolytic assays: i) the enzymatic activity of the crude enzyme used as a catalyst in organic media with unnatural substrates and ii) the stereopreference of the biocatalyst. To answer these questions, several reactions - using the lipases described in Table 1 - were performed. These reactions are repetitive and easy to do in an organic chemistry laboratory as reaction tests (see Experimental). The same batch enzymes were used in all the reactions so as to have the same biocatalyst in all the synthetic reactions.

2.3-Synthesis of heptyl oleate



Scheme 1.- Enzymatic synthesis of heptyl oleate.

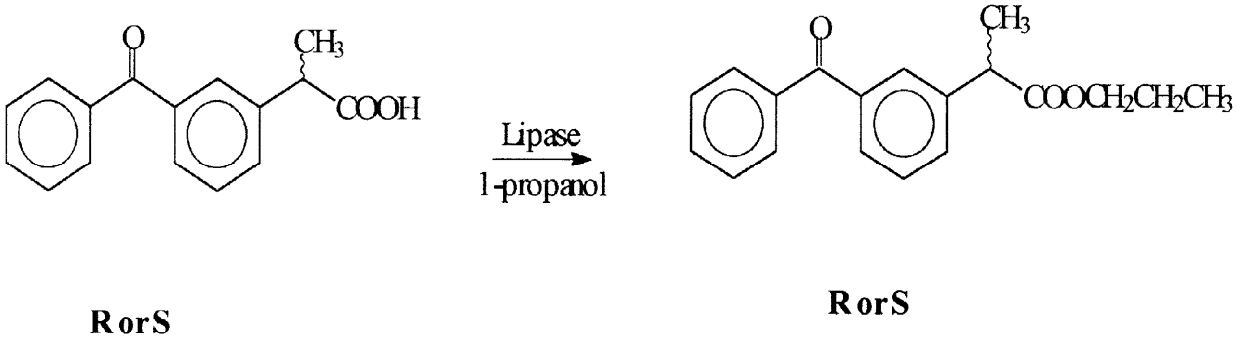
This reaction is performed in an organic medium (heptanol and oleic acid) but uses natural substrates of the lipase (alkanoic acid and alkanol). We recommend this reaction to differentiate an esterase or a protease (unreactive as a biocatalyst) from a lipase (excellent biocatalyst for the reaction). The results are shown in Table 2

Table 2.- Synthesis of heptyl oleate using lyophilized crude lipases

Inducer (%)	Yield of heptyl oleate after 24 h (%)
commercial lipase (L 85)	67
oleic acid (0.2%)	75
olive oil (0.5%)	85
sunflower oil (0.2%)	49
dodecanol (0.2%)	72

We observe that **the relative enzymatic activity in the synthesis of heptyl oleate in organic medium is not related to the lipase activity** (Table 1). The crude lipase obtained using dodecanol (0.2%) as inducer (lipase activity 1.04 U/ml , Table 1) gives a similar yield (72%) to the lipase obtained with oleic acid (0.2%) as inducer (lipase activity 2.5 U/ml). Thus, **the repetitive assay of hydrolysis is not useful for organic chemists to determine how much enzyme (Units or milligram) must be added to a reaction.**

2.4.- Enantioselective esterification of (R) or (S) Ketoprofen



Scheme 2.- Enantioselective esterification of (**R**) or (**S**) Ketoprofen.

The situation is more dramatic in the case of the esterification of the unnatural chiral acid (**R** or **S**)-2-(3-benzoyl)phenylpropionic acid (ketoprofen) in organic media. The relative catalytic activity observed in Table 2 for the synthesis of heptyl oleate is:

lipase (olive oil) > lipase (oleic acid) ≈lipase (dodecanol) > lipase (sunflower oil)

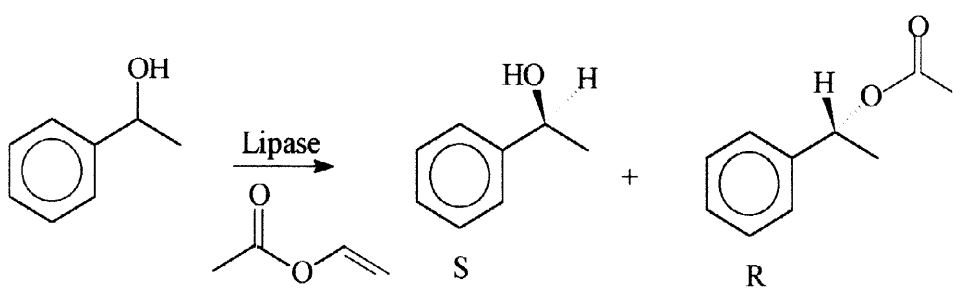
and is not reproduced with unnatural substrates.

Table 3.- Esterification of **R** or **S**-Ketoprofen

Inducer (%)	Yield (500 hrs) (%) **R**- ester	Yield (500hrs) (%) **S** -ester	**S/R**
commercial lipase (L 85)	2	17	85
oleic acid (0.2%)	11	13	122
olive oil (0.5%)	85	12	136

The **S**-enantiopreference described [5] for this enzyme remains unchanged in both cases but the yield and/or the enantioselectivity is very different. While commercial lipase (L-85) is relatively stereoselective (Table 3), the crude lipases obtained using oleic acid or olive oil as inducers are not stereoselectives (R/S ratios 1.2 and 1.3). The lipase obtained with olive oil as inducer (0.5%) (lipase activity 5.6 U/ml, Table 1) is less active than the lipase obtained using oleic acid as inducer (0.2%) (lipase activity 2.5 U/ml). Therefore we must conclude that the lipase hydrolytic activity used by the suppliers is not a good value for characterizing the lipase in synthetic reactions. However, the synthesis of an aliphatic ester such as heptyl oleate alone is not useful as the reaction test to predict the catalytic activity of the lipase from *Candida rugosa* using unnatural substrates. This is due to the presence of different percentages of isoenzymes LA and LB as discussed later.

2.5.- Enantioselective transesterification of (R,S)-1-phenyl-ethanol



Scheme 3.- Enzymatic transterification of (**R,S**)-1-phenyl-ethanol.

Table 4.- Enantioselelctive transesterification of (**R,S**) 1-phenylethanol

Inducer (%)	Ester yield (%)
commercial lipase (L 54)	74
commercial lipase (L 85)	56
oleic acid (0.2%)	37
olive oil (0.5 %)	16

In the transestefication of (**R,S**)-1-phenylethanol (Table 4), the most active crude lipase was the one with olive oil as inducer. This crude enzyme is more active than both commercial batches. Nevertheless, in all cases (commercial lots and our crude lipases), R-enantiopreference is observed (> 95 % e.e.). The fermenter conditions do not change the enantiopreference in this reaction [12]. The low reaction yields obtained agrees with the low reactivity observed for this enzyme with this type of secondary alcohols [13].

3. Discussion

The observed differences in the catalytic behaviour of the crude lipases as biocatalysts in synthetic reactions in water immiscible organic solvents must be related to three main factors:

i) presence of different percentages of the isoenzymes, lipases A (LA) and B (LB)

ii) different stability of both isoenzymes in organic solvents (lipase A is less stable than lipase B in isooctane, the solvent used in all the experiments [14])

iii) different percentage of water in the lyophilized crude enzymes. The amount of water is very important to control the flexibility of the protein molecule in the organic solvent and thus in the catalytic activity [15]. A rigid protein cannot catalyze a reaction in the slightly hydrated organic solvent [16].

From these points, only the first one can be important in hydrolytic conditions. However, the three points are very important to explain the catalytic activity in slightly hydrated organic media. To get deeper in the knowledge of these topics, the electrophoresis and FPLC analysis of the commercial lipase from Sigma (L 85) and of the crude lipases obtained by us in different fermenter conditions were obtained. The data are shown in Table 5.

Table 5.- Biochemical characterization of the crude lipases

Lipase and inducer	Isoenzyme A (%)	Isoenzyme B (%)
Commercial (L85)	80	20
Oleic acid (0.2%)	50	50
Sunflower (0.5%)	13	87
Olive oil (0.2%)	50	50
Dodecanol(0.2%)	50	50

We observe that while the commercial sample has a ratio of 4:1, our crude lipase has a ratio of 1:1, except for the crude lipase obtained using sunflower oil as inducer that shows a ratio of 1:5 for both peaks. The lipases produced using olive oil and dodecanol as inducers gave a ratio of 1:1 for the peaks (table 5). Depending on the fermenter conditions different relative proportions of isoenzymes LA and LB are produced.

Since the relative specific reaction rate constants for both isoenzymes in the hydrolysis of tributyrin are [17] LA 721 U/mg protein and LB 319 U/mg protein we could explain the different enzymatic activity observed in the

hydrolysis of this triglyceride. The lipase produced using sunflower oil as inducer shows very low lipase activity (Table 1) because it has low lipase A percentage.

The number of esterase units (U/ml Table 1) is greater than the number of lipase units. This is due to the presence of one esterase (43 kDa) and one protease (50 kDa) in the crude samples as described previously [18,19]. These enzymes can hydrolyze the p-nitrophenylacetate (water soluble substrate) but not the tributyrin (water insoluble substrate). Therefore the esterase activity is not a good method to define a lipase and so, it is not used by the suppliers.

The importance of water in the enzyme-catalyzed reactions in slightly hydrated organic medium is well documented in the literature [20,23]. Recently Gentili *et al.* [24] have shown that thermal analysis may be related to the enzymatic activity of lipases. In Figure 1 we show the thermogravimetric analysis (TGA) and the differential thermal analysis (DTA) of the commercial lipase (L85) and pure isoenzymes LA and LB.

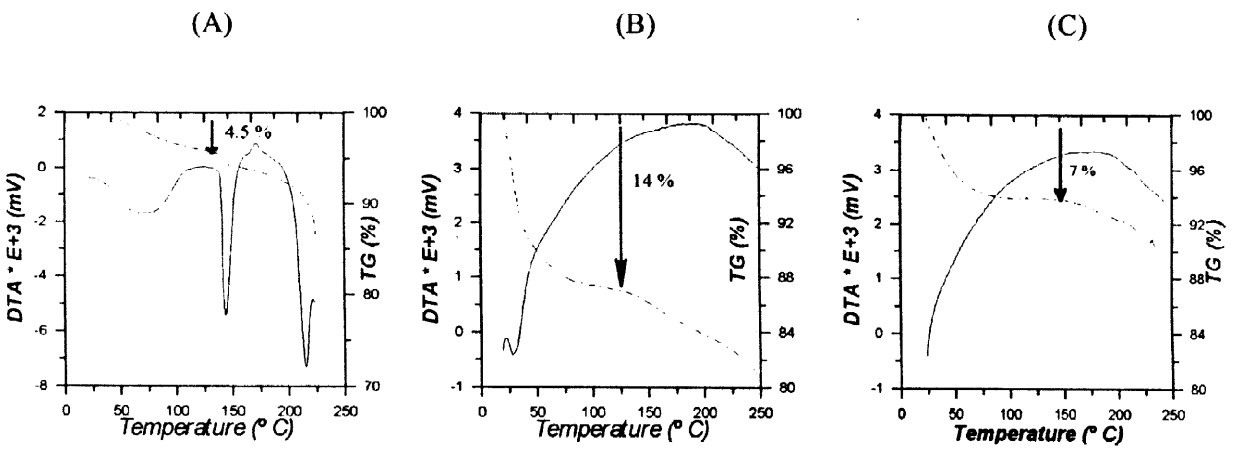


Figure 1.- Curves of TGA (- -) and DTA (–) of several lipases: A) Commercial lipase (L85), and pure isoenzymes, B) Lipase A and C) Lipase B.

Lipase from Sigma shows a weight loss between 23 and 135°C (4%, endothermic peak at 71°C) and the presence of lactose monohydrate added by the supplier as stabilizer (38% weight) [22].Both isoenzymes show a very different TGA/DTA profile. Lyophilized LA has 14% of water but it is not as strongly bonded to the protein as in the case of CRL because it is eliminated at T< 50°C. Lyophilized LB has only 7% water very weakly bonded because no endothermic peak associated with the water lost is observed. Depending on the relative percentage of lipases A and B the amount of retained water by the solid should be different . This fact is very important for explaining the enzymatic activity in slightly organic media because the solvent and the biocatalyst compete for the small amount of water present in the reaction mixture [20].

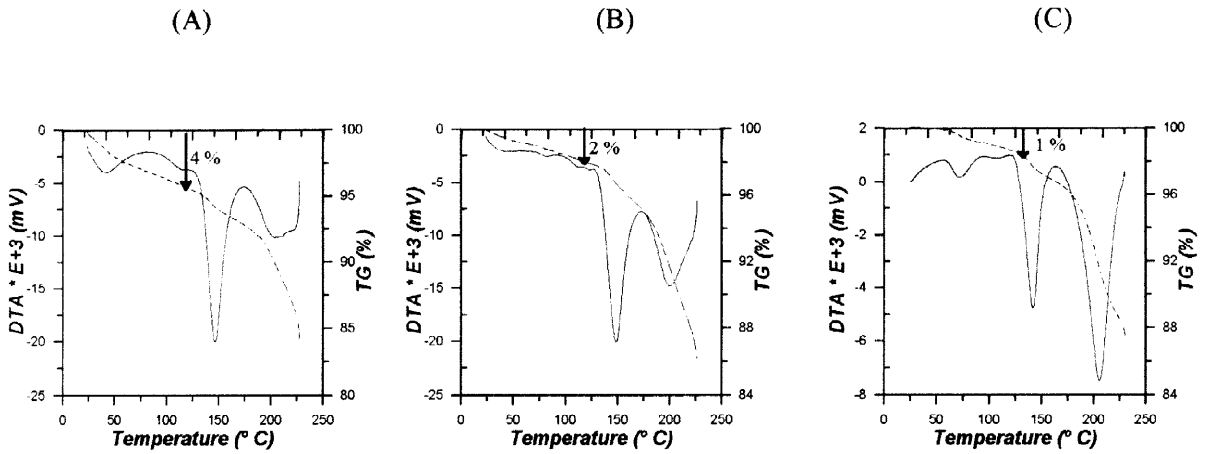


Figure 2.- Curves of TGA (- -) and DTA (-) for several lipases obtained in different inducers: A) Olive oil. B) Dodecanol. C) Oleic acid.

In Figure 2 we show the TGA/DTA curves of the main lyophilized crude lipases used in this paper. In the case of the lipase obtained using dodecanol (0.2%) as inducer (Figure 2b) only 2% water lost is observed (T<125°C). In the case of the lipase obtained using oleic acid as inducer, 1% of water lost (endothermic peak at 75°C) (Fig. 2c) and 4% water lost in the case of the lipase produced using olive oil (endothermic peak at 45°C) (Fig. 2a). The strong endothermic peaks observed at T>100°C could be related to dehydration of $(H_4N)PO_4 \cdot n\ H_2O$ produced in the fermenter conditions [25]. Depending on the inducer, the percentage of water in the lyophilized enzyme is different and so, the amount of water to be added to obtain the best yield in a lipase-catalyzed reaction in organic media will be different. This point has not been described until now. From the lipase-catalyzed synthesis that have been performed under the same experimental conditions in this paper, these differences are clearly showed in Tables 3 and 4.

The heptyl oleate synthesis (Table 2) is not sensitive to the different relative proportions of LA and LB in the crude lipase or to the amount of water in the lyophilized biocatalyst because they are natural substrates of lipases and therefore similar yields are obtained. Only lipase produced using sunflower oil as inducer shows a low yield probably due to the low percentage of LA (Table 5). The differences are more and more significant, as the unnatural substrates become more and more complicated.

In the esterification of pure (**R**) or (**S**) Ketoprofen, the differences are explained by the relative ratio LA/LB of the crude lyophilized lipase. LA is more S-stereoselective than LB in the case of Ketoprofen, due to steric reasons in the active site [17]. Sigma commercial lipase (LA/LB=4/1 Table 5) is more S-stereoselective than our crude lipases where the ratio is LA/LB=1/1 (see Table 3). In this case the percentage of water in lyophilized powder does not seem to be very important. This effect is not observed in Table 4 because both isoenzymes have R-stereopreference.

4. Conclusions

Lipase activity, measured as hydrolysis of tributyrin is a valid assay to quantify the lipase activity of a lyophilized crude lipase in hydrolysis reactions but it is not useful to predict the catalytic activity in lipase-catalyzed reactions in organic media. Three factors may influence the absence of reproducibility in the lipase-catalyzed reactions in slightly hydrated organic media using the lipase of *Candida rugosa*: i) relative proportion of isoenzymes; ii) amount of water in the lyophilized crude enzyme and iii) amount of lipase protein in the commercial powder. These factors may be controlled in each sample that arrives in the laboratory. In this way we propose two simple reaction tests :

i) heptyl oleate synthesis, specific of lipase . This reaction is not catalyzed by esterases or proteases that impurify the commercial lipase

ii) enantioselective esterification of (**R**) or (**S**) Ketoprofen. This reaction indicates the relative proportion of isoenzymes A and B in the commercial lyophilized enzyme (LA is more S-stereoselective than LB).

5. Experimental

5.1.- Materials.- Commercial lipase from *Candida rugosa* (Type VII) was obtained from Sigma Chemical Co. (St.Louis Mo.USA). (R) or (S)- 2-(3-benzoyl)phenylpropinic acid (Ketoprofen) was a gift from Menarini Laboraries (Barcelona, Spain). (R,S) 1-phenylethanol, arabic gum and *p*NPA were from Sigma Chemical Co (St.Louis Mo.USA). Dodecanol, n-propanol, tributyrin, NaCl, glycerin, isooctane and vinyl acetate were obtained from Aldrich. Oleic acid and n-heptanol were obtained from Merck.

*5.2.- Fermenter conditions.-*Fermentation experiments were carried out in a 6 l Braun fermenter Biostat E (4 l of working volume). The basal medium and the fermenter conditions were described by Obradors *et al.*[18]. Different inducers and percentages were used as carbon source: olive oil, oleic acid, sunflower oil and dodecanol. Extracellular crude lipase was obtained from the fermenter broth by centrifugation to remove the cells. The crude broth was concentrated and the crude lipase was obtained after lyophilization during 72 h.

5.3.- Esterase activity analysis.- The hydrolysis of *p*-nitrophenyl acetate (*p*NPA) as the substrate was followed spectrophotometrically at 25° C at 400 nm, monitoring the absorbance increase due to the *p*-nitrophenol release. The assay mixture (2.7 ml) consisted of 20 µl *p*NPA (10 mM) in acetonitrile, 2.5 ml buffer solution (0.1 M Tris/HCl, pH 7.5) and 50 µl of lipase solution. One unit of esterase activity is the amount of enzyme that liberates 1 µmol of *p*-nitrophenol per minute under the mentioned conditions.

5.4.- SDS PAGE.- SDS-PAGE was performed in 12% polyacrylamide gels, run on a Mini Protean II cell (Biorad Richmond, CA) and proteins were visualized by staining with silver according to the standard procedures.

5.5.- FPLC analysis.- A solution of 20mM Tris-HCl pH=7.4 buffer was used throughout the purification. A fast protein liquid chromatography system from Pharmacia, equipped with a column Memsep 1000 DEAE from Millipore was used for protein purification. The crude enzyme preparation (10 mg powder/ml) was applied to the column equilibrated with the buffer. The column was first washed with the buffer containing 0.3 M NaCl in order

to elute proteins without lipolytic activity.After that, two major peaks with lipolytic activity were eluted at NaCl concentration of 0.45 M and 1 M respectively [19].

5.6.- Lipase activity analysis.- Titrimetric assay was used to determine the lipolytic activity. This activity was monitorized in a Metrohm pH-stat (Impulsomat 614, Dosimat 665 (with microstirrer), pH-meter 691) (Herisau, Switzerland). A NaOH solution (0.025 M) was used as titrating agent. The reaction mixture consisted of different ml volumes of substrate emulsion (varying from 2 to 6 ml), several volumes of lipase solution (which ranged from 0.25 to 1 ml) and 0.1 M Tris/HCl buffer (pH 7.00), up to a total volume of 10 ml. In a typical procedure, Tris-HCl buffer (pH 7.00) and enzyme solution were placed in the thermostatically controlled cuvette of the pH-stat and maintained at 30° C. The mixture was shaken vigorously for 10 min. Stirring rate was adjusted to approximately 400 rpm and the substrate emulsion was added.

In all cases, lipase activity was measured as the initial reaction rate to avoid possible inhibition that might take place owing to the appearance of reaction products. The slopes of the initial linear stretch of the kinetic curves were determined graphically. Experiments were performed in duplicate. Maximum deviations from the mean were less than 5 %. Specific lipase activity was defined as the μmol of free fatty acids released per min and per ml of enzymatic extract.

5.7.- Synthesis of heptyl oleate.- The reaction mixture was composed of organic solvent (isooctane, 3 ml), 1-heptanol (20 mM), oleic acid (20 mM) and 0.3 ml of doubly distilled water. The reaction was carried out at a fixed temperature by shaking in 10 ml flasks for a specified time (24 h). The samples were analyzed by gas chromatography for conversion to the ester.

5.8.- Enantioselective esterification.- The standard reaction mixture was composed of isooctane (10 ml), R(-) or S(+) Ketoprofen (66 mM) and 1-propanol (264 mM). The reaction was carried out at 30° C by magnetic stirring in 25 ml-flasks. The reaction was started in all the cases by adding the same lipase units of treated lipases. The ester conversion was analyzed by HPLC [17].

5.9.- Enantioselective transesterification of (R,S)- 1-phenyl ethanol.- The standard reaction mixture was composed of isooctane (10 ml), (R,S)-1-phenylethanol (1 M) and vinyl acetate (1 M). The reaction was carried out at 30° C by magnetic stirring in 25 ml-flasks. The desired ester was analyzed by HPLC.

5.10.- HPLC analysis.- i) For esterification. The conversion and the e.e. were determined using a chiracel-OD column (Daicel Chemical Ind., Japan). The mobile phase for ketoprofen was hexane/2-propanol/acetic acid (90:10:1 by vol) of 0.5 ml/min. In the ibuprofen reaction, the mobile phase was hexane/2-propanol/ trifluoroacetic acid (100:1:0.1 by vol) of 0.8 ml/min at 25° C. The compounds were detected at 254 nm. ii) For transesterification. The conversion was determined using the same conditions with the exception to the mobile phase which was hexane/2-propanol (90:10 by vol) with a flow rate of 0.5 ml/min.

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