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Comparison of the Enzymatic Activity of Commercial and Semipurified Lipase of *Candida cylindracea* in the Hydrolysis of the Esters of (R,S) 2-Aryl Propionic Acids.

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Abstract: *A semipurified lipase of Candida cylindradea (LS) -easily obtained from commercial crude lipase (LC)- is used in the enantioselective hydrolysis of (R,S) 2-arylpropionates. The semipurification treatment diminishes the lipase activity more than the esterase activity. The addition of lactose (24 h) increases both activities. LS is more active than LC -at the same amount of protein- in the hydrolysis of (R,S) 2-aryl propionates. This semipurification showed a remarkable improvement in yield in the enantioespecific hydrolysis of these esters.*

Key Words: Lipase *Candida cylindracea*; semipurification of the enzyme; enantioselective hydrolysis; (R,S)2-arylpropionic acids resolution.

Lipases (EC 3.1.1.3) are enzymes which hydrolyze -in vivo- triacylglycerols to fatty acids and glycerol. These enzymes present low specificity with respect to the substrate. Therefore they are very useful for diverse synthetic purposes [1,2]. In this way commercial lipases have been used in some stereoselective organic procedures with good enantiomeric excess [3-6]. Nevertheless, the presence of at least two different isoenzymes -with different enzymatic activity profiles- and of glycopeptides that can present esterase activity have been reported [7-9]. Thus, a revision of the reported data should be carried out using purified or semipurified lipases. Recently, an easy semipurification methodology from impurities (M_w < 20.000) of the commercial lipase of *Candida cylindracea* has been reported. This purification is very easy and cheap and produces to an active enzyme. These lipases (LS) present very interesting properties, from applications in hydrolysis, to the synthesis of triglycerides and heptil oleate [10,11].

Therefore, we think that this semipurified enzyme (LS) could be an interesting alternative to the commercial lipase in organic synthesis because lower amounts of biocatalyst can be added to the reaction flask, and secondary reactions -due to the impurities the crude preparation- could be avoided without an increase in the cost of the biocatalyst. In addition, the lipase activity remains in LS, because both isoenzymes are present.

RESULTS AND DISCUSSION.

Semipurification of the lipase: the semipurification of commercial lipase by dialysis produced a weight decrease of 562 % related to the removal of low molecular weight molecules (Mw < 20.000). Consequently,

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the percentage of protein in the LS is greater (55 %) than in LC (22 %). The decrease observed in the number of Trp of LS with respect to the number of Trp in the LC (Table 1) sample must be related to the elimination of small peptides (M_w < 20.000) that would have Trp residues. Nevertheless, this number is greater than the number of Trp reported by Rua et al.[11] for pure lipase (5 Trp). Sánchez-Montero et al.[10] reported that dialysis removes the lactose molecules from the LC sample producing a diminution in the enzymatic activity, which could be restored by equilibration of LS with a lactose solution. In this way we have prepared LSL enzyme by equilibration of LS with a saturated solution of lactose for 24 h (see experimental).

Enzymatic activity: the enzymatic activity was measured in the hydrolysis of p-nitrophenyl-butyrate (esterase activity) and of olive oil (lipase activity) using the same amount of protein in all cases. We can observe in Table 1 that both lipase and estearase activities diminish after semipurification (LC versus LS) but increase when lactose is introduced in the enzyme (LS versus LSL).

Table 1. Influence of lactose in the esterase and hydrolytic activity (UI/mg protein) of different lipases of *Candida cylindracea.*

Lipase	Protein	Trp	Activity			
			Lipase IU/mg	% Remainig Activity	Esterase IU/mg	%Remainig Activity
LC	22	27	8.72	100	236.12	100
LSL	55	21	7.22	82.8	207.06	92.3
LS	55	21	4.74	54.3	187.10	83.7

Therefore, we can conclude that lactose plays an important role in the enzymatic activity, specially in the lipase activity. The diminution in the enzymatic activity produced by the elimination of lactose is not related to denaturation of the enzyme, but probably to a diminution of the specific activity of the enzyme molecules by the alteration of the hydrophylic shield, because when lactose is added (LSL) the enzymatic activity is partially restored. This affirmation is supported by the results of Sánchez-Montero et al.[10] that show: i) the enzymatic activity of LS can be restored at different levels depending on the nature of sugar reintroduced after the dialysis and ii) the removal of sugar molecules and of small peptides changes the microenvirommental dielectric constant of the lipase (negative at pH work pI=5.8) [9]. This fact difficults the interfacial mechanism of lipolysis or the monophasic process of the hydrolysis of esters.

The esterase activity decreases less than the lipase activity (Table 1). In addition, the introduction of lactose produces an increase in the esterase activity, 83.7% → 92.6% lower than in the case of lipase activity 54.3% → 82.8%. Similar results were reported by Sih et al. [7] after the purification of crude *Candida cylindracea* lipase on a SP-sephadex C-50 columm (crude lipase 71,958 units and SP-sephadex C-50 sample 44.650 units using olive oil as substrate). This modification removes the sugars as efficiently our methodology. In the case of esterase activity, there is not a rigid lipase oil/water interface interaction and the

microenvironmental alteration by the removal of lactose will not be as important as in the case of the lipase activity where the interaction with the oil/water interface is the rate controlling step .This interaction does not take place in the case of the esterase activity [12]. Therefore we might conclude that the small molecules (M_w < 30.000) non-covalently bonded to the protein, are important in the crude protein oil-water interface interaction.

The increase in the lipase activity observed after the introduction of lactose (LS v.s. LSL) should be explained in the sense of the restauration of the microenviroment of the lipase which favours the lipase interface oil-water interaction and thus, the lipase-activity increases. Nevertheless, the small peptides removed in the semipurification process (detected by the diminution in the Trp number (see Table 1)) and not introduced in the case of LSL, should be important in the lipase interface olive oil-water interaction because 100 % activity is not recovered. Therefore, we request from structural biochemists a more detailed analysis of these small molecules present in the crude lipase preparations to analyze their influence on the hydrolysis of olive oil.

The optimun pH value is 7.0, both in the presence and in the absence of external ions (Figure 1). This optimun pH value agrees with the results reported by other workers for LS [7,10,11] and for LC [13,14,15].

The positive effect of the ionic strength on the enzymatic activity of the commercial lipase using olive oil as subtrate has been reported in the literature [16,17,18]. In order to analyze this effect in the case of LS, the lipase activity was analyzed at different ionic strengths using two different buffers (Na_2HPO_4/NaH_2PO_4 and Tris/HCl both 0.1 M and pH=7.0) (Figure 2).

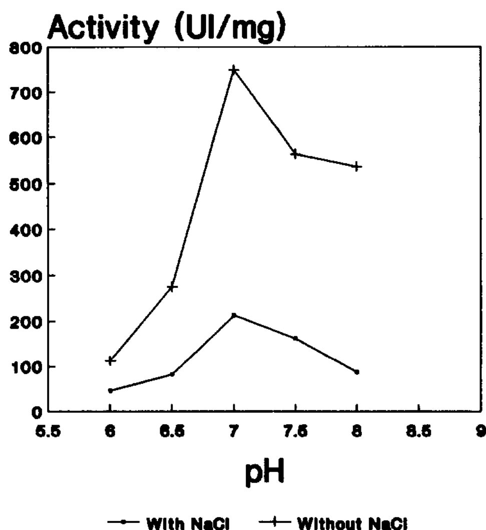


Figure 1. Influence of pH in presence and absence of ions.

The ionic strength was increased by the addition of NaCl. We can observe that the qualitative behaviour is the same both in LC and LS. Tris-HCl/NaCl buffer is better than Na_2HPO_4/NaH_2PO_4 buffer.

Therefore we should conclude that buffer ions interact with the protein. We are working on the analysis of this effect. To the best of our knowledge it is not described in the literature, where the enzymatic activity is generally determined in phosphate buffer pH= 7.0 [5,6,19] and in some cases in other buffers such as borate buffer (pH= 8.0) [20]. The increase in the ionic strength favours the lipase activity in both cases. This effect must be related to the removal of fatty acid molecules by Na(I), as sodium salts, and to the stabilization of the interface by the presence of ions. Therefore, the increase in the enzymatic activity due to the ionic strength-described for commercial lipase [17,20] -is observed in the case of LC.

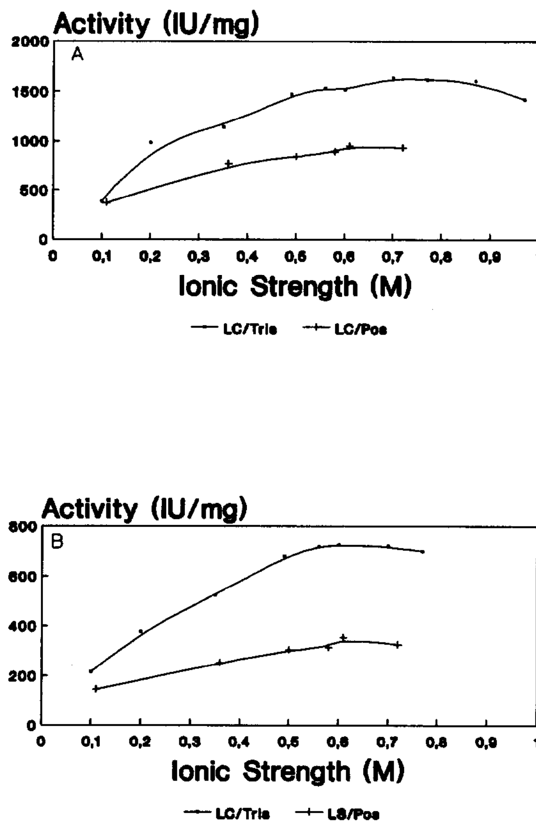


Figure 2- Influence of the nature of the buffer 0.1 M, on the lipase activity at 37 °C and pH=7.0; **A)** Commercial lipase: 1) ■ 0.1 M Tris/HCl; 2) + 0.1 M Na_2HPO_4/NaH_2PO_4. **B)** semipurified lipase 1) ■ Tris-HCl; 2) + Na_2HPO_4/NAH_2PO_4.

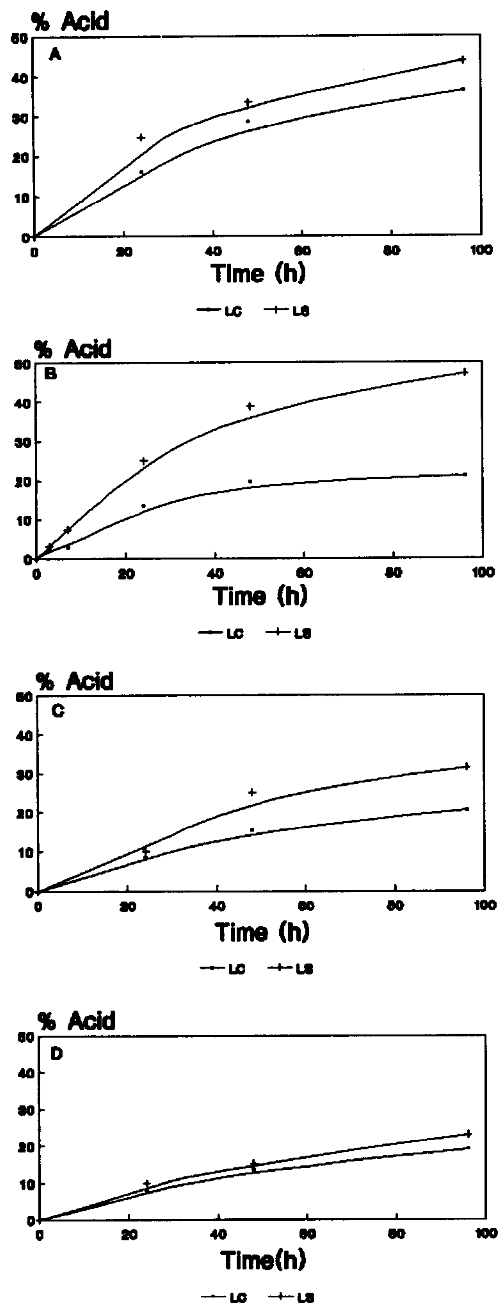
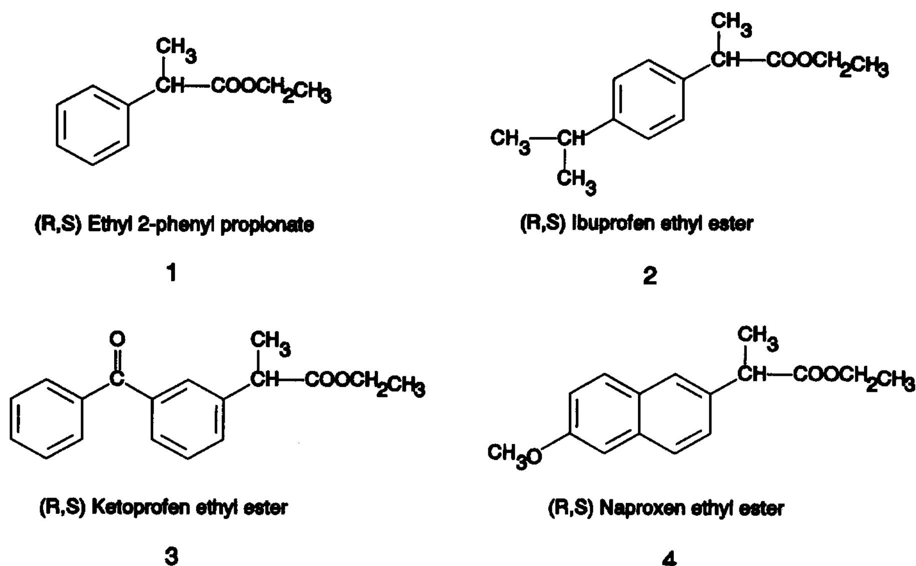


Figure 3. Hydrolysis of the esters with LS and LC **A)** (±) ethyl 2-phenylpropionate 0.1M **B)** (±) Ibuprofen ethyl ester 0.1M **C)** (±) Naproxen ethyl ester 0.02M **D)** (±) Ketoprofen ethyl ester 0.02M.

The hydrolysis of ethyl (R,S)-2-aryl-propionates.



The hydrolysis of these esters was carried out with both enzymes (Figure 3) using 0.1M Tris-HCl pH=7.0 buffer and not phosphate buffer as used by other workers [19], because both enzymes have greater enzymatic activity in this buffer (Figure 2). The pH value was 7.0 because this is the maximun activity value (Figure 1) and not pH=8.0 as reported by Sih et al. [19]. All the reactions were carried out with the same amount of protein [LC = 1.528 mg solid/ml (22 % of protein)= 0.336 mg prot/ml, LS = 0.6175 mg solid/ml (55 % of protein)= 0.339 mg protein/ml] but the severity of the process was different because different concentrations of substrate were used to obtain significative yield [(R,S) Naproxen and (R,S) Ketoprofen ethyl esters = 0.02 and methyl (R,S) 2-fenil propionate and (R,S) ibuprofen ethyl ester = 0.1 M]. These experimental conditions show us steric restrictions with the large molecules in the interaction enzyme-substrate. In addition, we should conclude that the esters do have not diffusional problems through the water to the oil-water interface because the enzymatic activity is not related to the lipidic properties of the ester molecule, (defined by the Hansch parameter π) (Table 2). Probably the large molecules of (R,S) ketoprofen and (R,S) naproxen ethyl esters present steric restrictions - due to the size of the aromatic ring- in the interaction with the active site of both lipases. This affirmation could explain to lower molarity of the substrate should be used with large molecules to obtain lower or similar yields at the same reaction times (Figure 3).

From Figure 3 we can deduce that LS is more active than LC at the same amount of protein. The explanation of the different enzymatic activity observed with respect to olive oil (LC > LS) and these esters (LS > LC) is very difficult. We could postulate that the nature of the lipidic part of the interface is different

in each case due to the presence either of alkyl chains in the olive oil (which only interacts by hydrophobic bands) or of the aromatic ring in the case of esters (which can interact both by hydrophobic bonds and by π-π interactions). This fact could give a different ordenation of the substrates in the interface and -more importantly- interaction with different aminoacids (Phe and Tyr in the second case) of the protein giving a different enzyme-sustrate interaction, more favorable with respect to the 2-arylpropionates in the case of LS than in LC. Our results are in agreement with the data reported by Sih et al. [19] in the hydrolysis of 2-aryl and 2-aryloxipropionates with crude lipase and purified lipase prepared by the authors, using deoxycholate and organic solvents. These authors show an increase in kcat/km value after the purification steps, which relate to a non-covalent modification of the enzyme. A similar explanation could be postulated in our case because only non covalently bonded small molecules have been removed from the crude protein by our method.

The lower percentage of (S) acid obtained with (R,S) Naproxen and (R,S) Ketoprofen ethyl esters than with (R,S) Ibuprofen ethyl ester and ethyl (R,S) phenylpropionate must be related to the steric restrictions of the esters of (R,S) Naproxen and (R,S) Ketoprofen with respect to the ester of (R,S) Ibuprofen and of (R,S) phenylpropionate ethyl ester. The lower percentage of (S) acid of the (S) 2-phenylpropionic acid with respect to (S) Ibuprofen acid must be related to the lipidic properties of the substrate, which can be described by Hansh parameter π because both acids have the same aromatic ring. This parameter has been calculated from the literature [20] using 2-phenylpropionic acid as reference compounds. The results obtained are shown in Table 2.

Table 2. Calculated π values for the substrates [20].

Substrate	π
1	0.00
3	2.94
4	3.08
2	4.29

Finally, in all cases the reaction of enzymatic hydrolysis was enantioespecific, but we have observed an increase in the porcentage of (S) acid obtained in the case of the LS with respect to native enzyme. In Figure 4 we show the ^{1}H-NMR spectrum (CH_3-CH- zone) of the complex of (R,R) 1,2-diphenyl-1,2-diamino ethane with the 2-phenyl propionic acid produced in the hydrolysis of its ethyl ester with LS (Figure 4.a) and the ^{1}H-NMR spectrum of the racemic (R,S) 2-phenyl propionic acid (Figure 4.b). Similar results are obtained in all cases (see Table 3). Therefore LS is more interesting as biocatalyst than LC in this reaction because, while presenting the same e.e., it gives greater yields at the same reaction time.

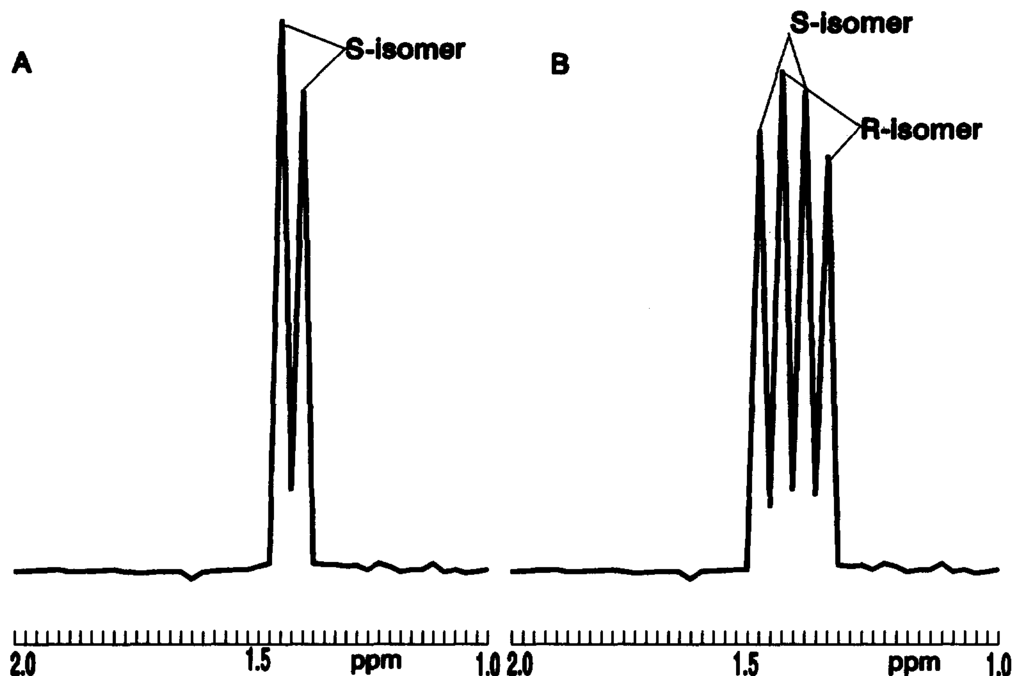


Figure 4. Increase in the stereoselectivity of the hydrolysis 2-phenyl-propionic esters: **A)** the product (S) **B)** the racemic mixture (R and S). For experimental details see Experimentals.

Table 3. The enantioselectivities of LC and LS.

Substrate	Enzyme	% Acid	Time (h)	e.e.	Stereo-preference
1	LC	30.52	96	>98 %	S-(+)
2	LC	21.18	96	>98 %	S-(+)
3	LC	19.14	96	>98 %	S-(+)
4	LC	20.53	96	>98 %	S-(+)
1	LS	43.70	96	>98 %	S-(+)
2	LS	47.24	96	>98 %	S-(+)
3	LS	23.04	96	>98%	S-(+)
4	LS	31.40	96	>98 %	S-(+)

EXPERIMENTALS

Lipase (E.C.3.1.1.3) from *Candida cylindracea* (Type VII), bovine serum albumin, guanidium chloride and the alcohols were obtained from Sigma Chemical Co. (St Louis, MO, USA). Olive oil emulsion (50:50, v/v) and silica gel was supplied by Merck Chemical. Racemic 2-phenyl propionic acid was purchased from Fluka (Buchs, Switzerland). Racemic ibuprofen was a gift from Boots Pharmaceuticals (Nothingham, U.K.), racemic ketoprofen from Menarini laboratories (Badalona, España) and racemic naproxen from Syntex Research (Palo Alto, California, U.S.A). All other chemicals were of the purest grade available.

Enzyme preparations. Three different enzyme preparations have been used in this work:
i) Commercial lipase (LC). ii) Semipurified lipase (LS) was obtained by dialysis in order to remove lactose and small molecules from the enzymatic preparation. Powdered lipase was mixed with double-distilled water and transferred to a dialysis bag. The bag was submerged in a vessel containing double-distilled water and mechanically stirred at 4°C. After 24 h of dialysis the enzymatic preparation was lyophilized for 48 h using a TELSTAR (model Liobeta) freeze dryer [10]. iii) Addition of lactose to dialysed enzymatic preparations. The lactose was added to the 'purified' lipase preparation by dialysis. In a typical experiment, 0.08 g of 'purified' lipase was mixed with 15 ml of double-distillated water and placed in a dialysis bag. The bag was submerged in a vessel containing a solution of lactose (5 g/ml) and mechanically stirred at 4°C for 24 h. The lipase mixture was then lyophilized as described above (LSL) [10].

Determination of Trp. Spectroscopic determination of tryptophan in lipase from *Candida cylindracea* was measured by the Edelhoch methods [21]. The ratio of the numbers of Trp and Tyr residues in a protein may be determined from the absorbances at 288 nm in neutral 6M guanidium chloride using the formula:

$$\frac{Trp}{Tyr}=\frac{A_{280*1280}-A_{280*385}}{A_{280*4812}-A_{288*5690}}$$

To a 0.8 ml of guanidium chloride 6M solution were added 200 μl of protein solution. The absorbance was measured in an UV-visible spectrophotometer Shimadzu 2100.

Protein measures. The amount of protein was determined by the colorimetric Hartree Methods [22].

Enzyme assays. Lipase activity was monitorized in a Crisson pH-stat (Micrott 2022, Microbur. 3031 and Microstirrer 2038). In a typical experiment, 7 ml of 0.1 M buffer solution (Tris-HCl or Na_2HPO_4-NaH_2PO_4) and 1 ml the lipase solution (8 mg/ml or 2 mg/ml of LC and LSL or LS respectivily) was introduced in the appropriate buffer solution in thermostated cuvette of the pH-stat. The mixture was vigorously shaken for 10 min at 37 °C. Then 2 ml of olive oil (0.1137 mg/ml in 0.1 M appropiate buffer solution) were added.
The hydrolysis of olive oil was carried out in the presence of the different buffers and/or ionic strengths. Experiments was performed in three times, and the maximun deviation from the mean were less than 5%. One unit (IU) of lipase activity is defined as the amount of enzyme needed to produce 1 μmol of oleic acid per hour at 37 °C.

The esterase activity using p-nitrophenyl butyrate as substrate, was followed spectrophotometrically at 35 °C in a Shimadzu 2100 spectrophotometer. The assay mixture (2.45 ml) consisted of 25 μl of p-nitrophenilbutyrate (1.5 μg/ml) in acetonitrile, 2.4 ml of buffer solution (Tris-HCl 0.1 M, pH=7.0) and 25 μl of lipase solution (0.04 mg/ml or 0.01 mg/ml of LC and LSL or LS respectivily). Initial reaction rates were estimated by measuring the increase in the absorbance at 400 nm. One unit (U) is the amount of enzyme that liberates 1 μmol of p-nitrophenol per minute under the above conditions.

Synthesis of esters. The ethyl esters of (R,S) 2-phenyl propionic acid, (R,S) naproxen, (R,S) ibuprofen and (R,S) ketoprofen were prepared by the classical methodology using thionyl chloride and ethanol. Thionyl chloride, 3 ml (0.04 mol), were adeded dropwise to a cooled, stirred suspension of 2-arylpropionic acid (0.024 moles) in ethanol (50 ml). The reaction mixture was refluxed for 2h. Then the solvent was evaporated and the residue purified by columm chromatography using SiO_2 as adsorbent an dicloromethane as eluant.

^{1}H-NMR spectra were taken with a Varian VXR-300 NMR spectrometer using $CDCl_3$ with TMS as internal standard. IR spectra were with Buck Scientific 500 spectrophotometer.

(R,S) Ethyl 2-phenylpropionate. **1.** Elemental analysis; Found: C, 74.02 %; H, 7.99 %. Calc for $C_{11}H_{14}O_2$: C, 74.13 %; H, 7.92 %. ^{1}H-NMR ($CDCl_3$, δ): 7.3-6.9 (m, 5H), 3.9 (q, 2H), 3.4 (q, 1H), 1.25 (d, 3H), 0.9 (t, 3H). IR (ν_{max} cm^{-1}): 3100, 3060, 2990, 1736.

(R,S) Ethyl 2-(4-isobuthylphenyl)propionate. **1.** Elemental analysis; Found: C, 76.98 %; H, 9.5 %. Calc for $C_{15}H_{22}O_2$: C, 76.8 %; H, 9.46 %. ^{1}H-NMR ($CDCl_3$, δ): 7.35-7.0.5 (m, 4H); 4.15 (q, 2H); 3.6 (q, 1H); 2.05 (d, 3H); 1.8 (m, 1H); 1.1 (t, 3H); 0.8 (t, 6H). IR (ν_{max} cm^{-1}): 3090, 3040, 2780, 1730.

(R,S) Ethyl 2-(6-methoxy-2-naphthyl)propionate. **1.** Elemental analysis; Found: C, 74.29 %; H, 7.12 %. Calc for $C_{16}H_{18}O_3$: C, 74.4 %; H, 7.02 %. ^{1}H-NMR ($CDCl_3$, δ): 7.75-7.1 (m, 6H); 4.20 (q, 2H); 3.9 (s, 3H); 3.8 (q, 1H); 1.56 (d, 3H); 1.2 (t, 3H). IR (ν_{max} cm^{-1}): 3100-2800, 1740.

(R,S) Ethyl 2-(3-benzoilphenyl)propionate. **1.** Elemental analysis; Found: C, 75.5 %; H, 6.73 %. Calc for $C_{18}H_{18}O_3$: C, 76.6 %; H, 6.38 %. ^{1}H-NMR ($CDCl_3$, δ): 7.54-7.12 (m, 9H); 4.15 (t, 2H); 3.65 (q, 1H); 1.4 (d, 3H); 1.0 (t, 3H). IR (ν_{max} cm^{-1}): 3070-2970, 1740.

Hydrolytic reactions of the esters of (R,S)-2-arylpropionic. Hydrolysis of (R,S) ethylic ester of arylpropionic acids was carried out as follows: to 4 ml of 0.1 M Tris-HCl buffer, pH=7.0 were added to 117 mg of (R,S) ibuprofen or 89 mg of (R,S) 2-phenylpropionic or 28 mg of (R,S) ketoprofen or 25 mg of (R,S) naproxen (0.1 M, 0.1 M, 0.02 M and 0.02 M reapectivily) and 1 ml the lipase solution (17.16 mg/ml) in the Tris-HCl buffer. The mixture was incubated at 30°C for 96 h under gentle stirring. The reaction was stopped by the addition of HCl (0.1 M). Sample periodically (0, 3, 7, 24, 48, 96 h) were withdrawn and analyzed by gas chromatography.

Gas chromatography Analysis. Gas chromatography was performed in a Shimadzu GC-14 A gas cromatography equipped with FID detector, a split inyector (1:2) and a SPB-1 sulfur column (15 m × 0.32 mm). Injector temperature was 300°C and detector temperature 350°C; carrier gas was nitrogen. Different conditions for quantitative analysis were used depending on the componds: for 2-phenyl propionic acid, a column temperature of 180° C and a N_2 stream of 3 ml/min; for ibuprofen a column temperature of 180° C and a N_2 stream of 12 ml/min; for naproxen and ketoprofen a column temperature of 190°C and a N_2 stream of 30 ml/min. An external standard method was employed to quantify the remanent ester and the formed acid.

Enantiomeric excess determination. The acid and ester were extracted from the reaction mixture with diethyl ether (3 × 25 ml). The organic phase was placed in a clean glass and a new extraction was carried out with NaOH 0.1 N (3 × 25 ml). The aqueous phase was acidified with HCl and then, a third extraction was performed with diethyl ether (3 × 25 ml). The ramaining organic phase was evapored to dryness. A solution 0.053M of the obtained residue (acid) and 0.027 M of (R,R)1,2-diphenyl/diaminoethane in 0.9 ml of $CDCl_3$ gave the diasteroisomeric salt complexes that allowed the direct ^{1}H-NMR determination (Brucker 250 ^{1}H-NMR) of the enantiomeric purity as described by Fullwood et al. [23]. The enantiomeric excess of the acid was checked by polarimetric methodology using chloroform as solvent.

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