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Hydrolysis of (*R,S*)2-aryl propionic esters by pure lipase B from *Candida cylindracea*

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Abstract

Purified lipase B from *Candida cylindracea* (LB) has been obtained in large amounts. LB exhibits greater esterase and lipase activities than commercial lipase. The presence of divalent and/or monovalent cations increases the lipase activity with respect to the absence of external cations, using olive oil as substrate. LB is more active than commercial and semipurified lipases in the hydrolysis of (*R,S*)2-arylpropionic ethyl esters. The presence of Na(I) or Ca(II) diminishes the enzymatic activity in the hydrolysis of these esters compared to that obtained in the absence of the external ions. LB is stereospecific in the hydrolysis of *S*(+)2-arylpropionate.

Keywords: Arylpropionic acids; *Candida cylindracea*; Enantioselectivity; Hydrolysis; Lipase; Propionic acids ; Purification

1. Introduction

Lipases (EC 3.1.1.3) are particularly suitable for organic synthesis, because they have a broad substrate specificity and have no coenzyme requirement for catalysis. Although the natural substrates of lipases are acyl glycerols, they can also catalyze the hydrolysis of a wide range of unnatural water-insoluble esters with a high degree of enantioselectivity. Therefore, commercial crude lipases have been used in the high enantioselective preparation of *S*(+)-2-arylpropionic acids by hydrolysis of esters or by esterification reaction [1–3].

The lipase produced by *Candida cylindracea* has been broadly used, owed to its high activity in hydrolysis [4] as well as in synthesis [5].

Since 1966, when Tomizuka et al. [6] purified and characterized a single extracellular lipase from *Candida cylindracea*, many papers have reported the multiple forms of lipases produced by microorganisms [7,8]. This multiplicity has been ascribed to either post-transcriptional process such as proteolysis [9], deglycosylation [7], or synthesis of different lipases by the same microorganism [10].

In spite of one lipase gene identified in *Candida cylindracea*, then cloned, sequenced and the amino acid sequence of the protein deduced from, the cDNA [11], at least two main lipases have been identified in extracellular cultures of the yeast [7–12]. Recently Alberghina et al. [13] have reported the presence of four isoforms for LB with the same N terminal sequence. These isoforms are not easily separated by conventional media and they are used as LB. These isoenzymes

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[lipase A (LA) and lipases B (LB)] have similar molecular weights (LA = 64 and LB = 62 kDa) but different isoelectric points (LA = 5.5 [14] and LB = 4.8–5.04 [13]) and different percentage of sugar (LA = 8% and LB = 3.6 wt% [14]). As a consequence, the isoenzymes have different hydrophobicity and enzymatic activity. LA is a more active esterase than LB, but LB is a more active lipase than LA [14].

In the present paper we show the first results obtained in the hydrolysis of non-acyl glycerol derivatives by LB. The hydrolysis of ethyl (*R,S*)-2-arylpropionates has previously been studied with the commercial lipase of *Candida cylindracea* [1–3] resulting in good yields and enantiomeric excess. We compare the enzymatic activity of commercial lipase with the most active and main component of LB, present in the crude powder. In addition, the enzymatic activity is compared with that of a semipurified lipase of *Candida cylindracea* (LS) obtained by dialysis of LC according to an experimental procedure described previously [15].

2. Experimental

2.1. Materials

Commercial lipase (E.C.3.1.1.3) from *Candida cylindracea* (Type VII, containing 1.0104 U/mg solid using olive oil as substrate), lipase substrate (stabilized olive oil emulsion 50% v/v), bovine serum albumin, guanidine hydrochloride and the alcohols were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Silica gel was supplied by Merck Chemical. (±)2-Phenyl propionic acid was purchased from Fluka (Buchs, Switzerland). (±)Ibuprofen was a gift from Boots Pharmaceuticals (Nottingham, UK), (±)ketoprofen from Menarini Laboratories (Badalona, Spain) and (±)naproxen from Syntex Research (Palo Alto, CA, USA). DEAE–Sephacel, Sephacryl HR100, Phenyl-Sepharose CL-4-B were obtained from Pharmacia (Sweden). All other chemicals were of the purest grade available.

2.2. Semipurification

Semipurified lipase (LS) was obtained by dialysis from the crude LC according to the procedure described by Sánchez-Montero et al. using a cut-off membrane of 20,000 [14].

2.3. Purification of lipase B

25 g of crude powder were suspended in 250 ml of 0.25 M phosphate buffer pH = 7 and 0.02% sodium azide, kept stirring for 90 min and centrifuged at 12,000 rpm for 20 min at 4°C. The clear solution obtained was loaded on a DEAE–Sephacel column (5 × 60) equilibrated with the same phosphate buffer. First eluted the material strongly coloured, without lipase activity and then eluted LB with phosphate buffer 1 mM, pH = 7, 0.02% sodium azide at 240 ml/h, 4°C. Fractions of 7.5 ml were collected and measured for absorbance at 280 nm for protein concentration, esterase activity at 400 nm (using *p*-nitrophenyl butyrate as substrate) and lipase activity (using olive oil emulsion). The fractions with high activity were separately pooled and concentrated by ultrafiltration through Amicon PM 30 membranes. Concentrated aliquots of LB were separately loaded on Sephacryl HR 100 column (2.6 × 80 cm) equilibrated with 0.1 M phosphate buffer pH = 7, containing NaCl 0.1 M and eluted with the same buffer at 20 ml/h, 4°C. Fractions of 4 ml were collected and measured for absorbance at 280 nm, esterase activity (using *p*-nitrophenyl butyrate as substrate) and lipase activity (using olive oil emulsion) [16].

2.4. Determination of tryptophan residues

Spectrophotometric determination of tryptophan residues in lipase from *Candida cylindracea* was carried out by the Edelhoch methods [17]. The ratio of Trp to Tyr (N_{Trp}/N_{Tyr}) residues in the protein can be determined from the absorb-

ances, at 288 nm, in neutral 6 M guanidine hydrochloride using the formula:

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

To a 0.8 ml of guanidine hydrochloride 6 M solution, 200 μl of protein solution were added. The absorbance was measured in a Shimadzu 2100 UV–visible spectrophotometer.

2.5. Protein concentration

The amount of protein was determined by the colorimetric Hartree methods [18].

2.6. Enzyme assays

Lipase activity was monitored in a Crisson pH-stat (Micrott 2022, Microburet 3031 and Microstirrer 2038). In a typical experiment, 7 ml of 0.1 M buffer solution (Tris–HCl) and 1 ml the lipase solution with the same protein concentration in all experiments (0.1138 mg protein), was introduced in the appropriate buffer solution into the 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 appropriate buffer solution) were added to the solution.

The hydrolysis of olive oil was carried out in the presence of the different cations (Na^+, Ca^{2+}, Na^+/Ca^{2+}) with different ionic strength. Experiments were repeated three times, and the maximum deviation from the mean values was 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.

2.7. Hydrolysis of ethyl (±)-2-arylpropionates

Synthesis of esters

The ethyl esters of (±)2-phenylpropionic acid, (±)naproxen, (±)ibuprofen and (±)ketoprofen were prepared by the classical methodology using thionyl chloride and ethanol. Thionyl chloride 3 ml (0.024 mol) were added dropwise to a cooled, stirred suspension of 2-arylpropionic acid (0.024 mol) in ethanol (50 ml). The reaction mixture was refluxed for 2 h. Then the solvent was evaporated and the residue purified by column chromatography using SiO_2 as adsorbent and dichloromethane 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 recorded on a Buck Scientific 500 spectrophotometer. The physical and spectroscopic constant of the esters were compared to those described in the literature.

Enzymatic hydrolysis

The hydrolysis of (±)ethyl ester of arylpropionic acids was carried out as follows: 4 ml of 0.1 M Tris–HCl buffer, pH = 7.0 were added to 117 mg of racemic ibuprofen, or 89 mg of 2-phenylpropionic, or 28 mg of ketoprofen and 25 mg of naproxen (0.1 M, 0.1 M, 0.02 M and 0.02 M respectively). Then 1 ml of the lipase solution (17.16 mg/ml) in the Tris–HCl buffer was added to the reaction vessel. 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). Periodically (0, 3, 7, 24, 48, 96 h) samples were withdrawn and analyzed by GC in a Shimadzu model GC-14 A gas chromatograph with FID detector, a split injector (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 nitrogen. Different conditions for quantitative analysis were used depending on the compounds: for 2-phenylpropionic acid; isotherm program $T = 180$°C and a N_2 flux of 3 ml/min; for ibuprofen an isotherm program $T = 180$°C and a N_2 flux of 12 ml/min; for naproxen and ketoprofen an isotherm program $T = 190$°C and a N_2 flux of 30 ml/min.

The hydrolysis in the presence of the cations with LB was carried out using NaCl and $CaCl_2$ 0.125 M [19].

2.8. Enantiomeric excess determination

The acid and ester were extracted with diethyl ether (3 × 25 ml) from the reaction mixture. The

organic phase was placed in a clean bottom flask and a new extraction was carried out with NaOH 0.1 N (3×25 ml). The aqueous phase was acidified with HCl and extracted with diethyl ether (3×25 ml). The remaining organic phase was evaporated to dryness.

A solution 0.053 M 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 diastereoisomeric salt complexes that allowed the direct ^{1}H-NMR determination (Bruker 250 ^{1}H-NMR) of the enantiomeric purity as described by Fullwood et al. [20]. The enantiomeric excess of the acid was checked by polarimetry.

3. Results and discussion

3.1. Lipase activity of LB

Lipase and esterase activities, measured with olive oil emulsion and *p*-nitrophenyl butyrate, respectively, have been determined with commercial (LC), semipurified (LS) and purified lipase (form B) (LB). As shown in Table 1, the lipase and esterase specific activities for the semipurified enzyme (LS) are lower than for the commercial enzyme (LC). Pure lipase B shows the highest esterase and lipase activities. Lactose plays an important role in the enzymatic activity, especially in the lipase activity, because in the semipurification procedure, lactose and small molecules (M_w lower than 20,000 Da [15] are removed from LC to obtain LS. The diminution in the enzymatic activity produced by the elimination of lactose is not related to denaturation of the enzyme, but probably to a decrease of its specific activity by the modification of the hydrophilic shield. As a matter of fact, when lactose is added (LSL) the enzymatic activity is partially restored, similarly results reported by Sánchez-Montero et al. [15]. The removal of impurities after purification improves the enzymatic activity by solid weight unit and enhances the specific lipase and esterase activities.

Previously we have reported that the lipase activity is increased by the presence of inorganic cations [19]. Therefore, we carried out the hydrolysis of olive oil at different values of ionic strength. The results are shown in Fig. 1. We observed similar qualitative behaviour in all cases but the effect is greater in the less purified enzyme LC than in pure enzyme (LB). This finding is related to the specific enzymatic activity, higher in LB than in LC. The positive effect of Na(I) and/or Ca(II) in the lipase activity in microemulsion is well documented [21–23], and it is related to the stabilization of the interphase by Na(I) and to the removing of two carboxylate molecules per Ca(II) from the oil/water interphase [24,25]. Nevertheless, the reaction sequence does not seem to be altered by the presence of ions, specially Ca(II), as has been demonstrated by Otero et al. [21] (Scheme 1).

These authors showed that Ca(II) increases k_3 but not k_2/k_1. Nevertheless, this is not a general mechanism because Hang et al. [25] have observed inhibition produced by Ca(II), Co(II) and Mg(II) in the hydrolysis catalyzed by LC in reverses micelles, but not by monovalent cations such as Na(I) or NH_4^+. These differences can be explained by the structure of the systems: (i) microemulsion oil/water and (ii) reverse micelles (Scheme 2).

In our case microemulsion oil/water, the reaction product (carboxylate molecule) is carried

Table 1
Influence of the degree purification in the lipase and esterase activity (IU/mg) of different lipases of *Candida cylindracea*

Lipase	Protein (%)	Trp	Lipase activity (UI/mg)	Esterase activity (UI/mg)
LC	22	27	8.62	236.12
LS	55	21	5.07	187.06
LSL	55	21	7.22	207.06
LB	100	5–9	92.47	15,500.00

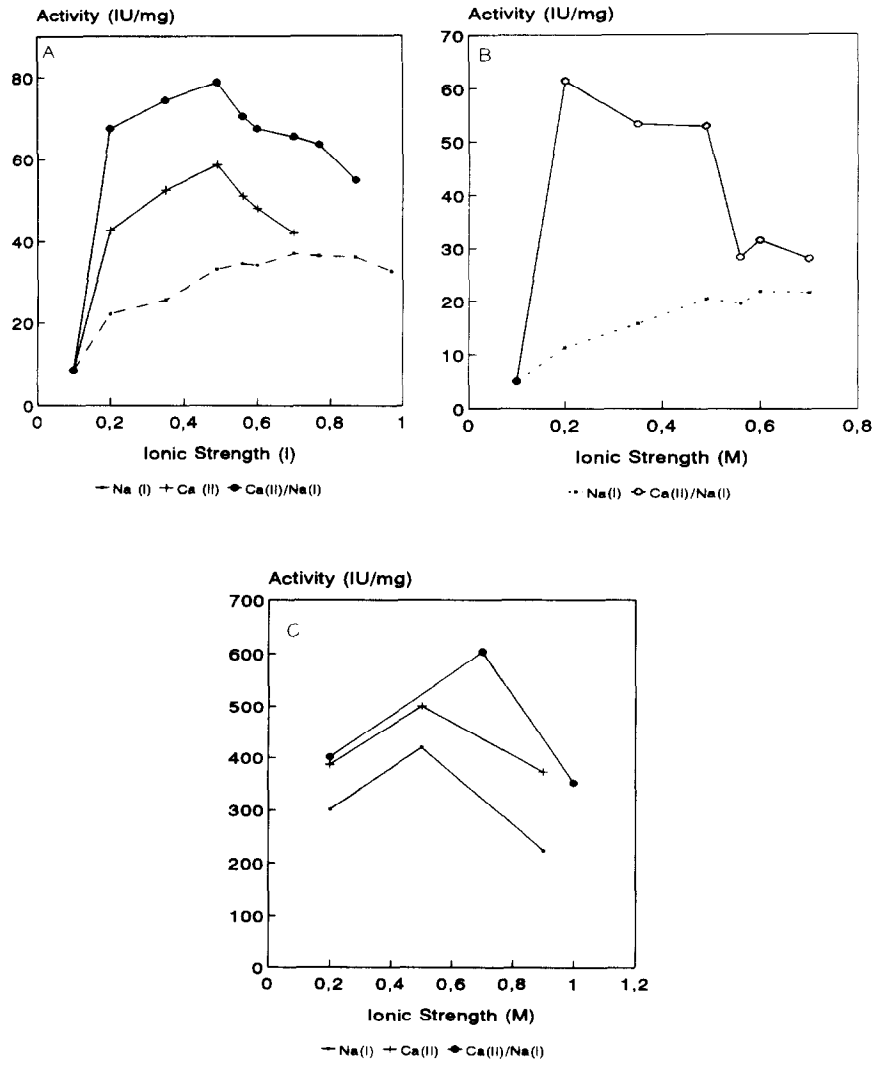
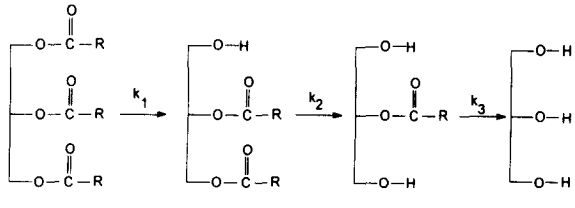
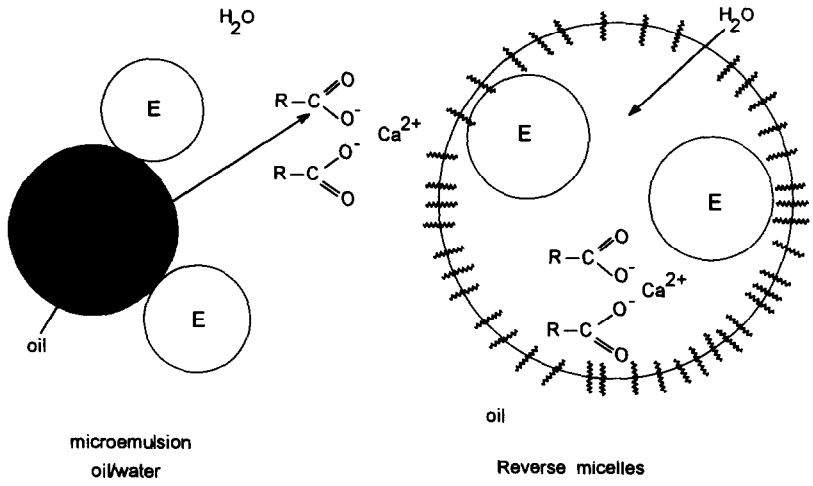


Fig. 1. Influence of the ionic strength and of the nature of the cation in the enzymatic activity of the lipases (the different cations are added in increasing concentration). (A): LC [■ Na (I), +Ca (II) and ○ Ca (II)/Na (I)]. (B): LS [■ Na (I) and ○ Ca (II)/Na (I)] (C): LB [■ Na (I) and ○ Ca (II)/Na (I)].



Scheme 1.

over in the microenvironment by the effect of Ca(II), avoiding the inhibition of the enzyme by the substrate. In the case of reverse micelles, those molecules are carried over into the micelle (water) producing enzyme inhibition by the presence of the reaction products [22]. Therefore, the positive or negative effect of the cations in the lipase activity is related to the interphase phenomenon and not to the true catalytic process.



Scheme 2.

We can observe similar behaviour with all enzymes LC, LS and LB. The presence of Ca(II) improves the enzymatic activity compared to the presence of Na(I). The maximum yield obtained only in the presence of Na(I), is achieved at higher ionic strength values compared to that in the presence of either Ca(II) or Ca(II)/Na(I). This finding could be accounted for by the ability of Ca(II) ions to remove the oleic acid molecules from the interphase and then, increasing the turnover number and by the ability of the Na(I) ions to stabilize the oil–water interphase. This assumption would be supported by the maximum enzymatic activity achieved in LC and LS at [Na(I)] = 2[Ca(II)]. Each Na(I) removes one carboxylate molecule and each Ca(II) removes two carboxylate molecules. The high specific activity of LB can explain the small effect of these ions in the lipase activity.

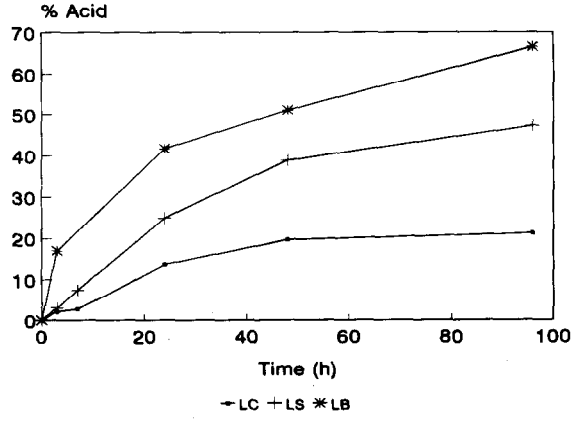
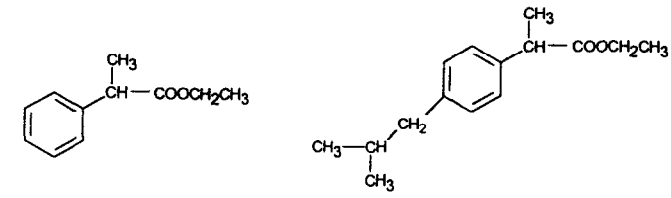


Fig. 2. Hydrolysis of Ibuprofen ethyl ester 0.1 M with LC (■), LS (+) and LB (*) of *Candida cylindracea* (30°C, buffer Tris–HCl 0.1 M pH = 7.0, time 96 h).

3.2. *Hydrolysis of ethyl (R,S)2-arylpropionates*

The hydrolysis of these non steroidal antiinflammatory drugs was carried out using the lipase *Candida cylindracea* at three different purification degrees. Fig. 2 shows the results obtained by testing LC, LS, and LB to prove their ability to catalyze the stereoselective hydrolysis of racemic ibuprofen ethyl ester. The stereoselective hydrolysis of racemic naproxen, ketoprofen and 2-phenylpropionic ethyl esters were only carried out with LC and LB. In addition, the effect of the cations in the reaction yields was analyzed. Thus, the hydrolysis of the substrates were carried out in the presence of external ions: 0.1 M Tris–HCl buffer, pH = 7.0 and 0.125 M NaCl/0.125 M $CaCl_2$ (LBC = Lipase B with cations).

The results are shown in Fig. 3. In all cases, the experiments were performed using the same concentration of active protein (0.1716 mg/ml), taking into account that only 22% of LC [26], 55% of LS [26] and 100% of LB are protein [19].



(R,S) Ethyl 2-phenyl propionate

1

(R,S) Ibuprofen ethyl ester

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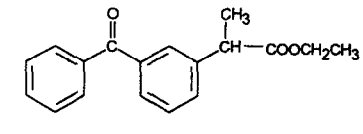

(R,S) Ketoprofen ethyl ester

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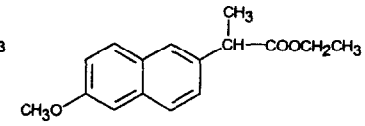

(R,S) Naproxen ethyl ester

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Scheme 3.

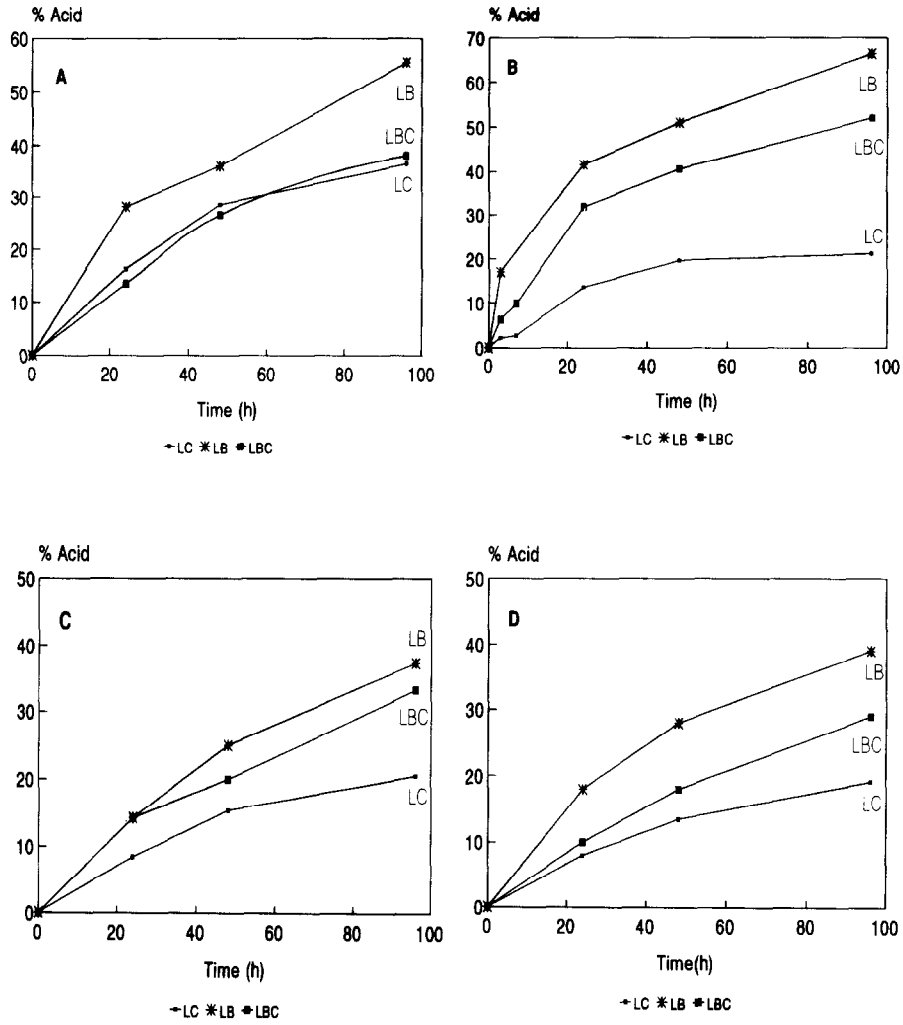


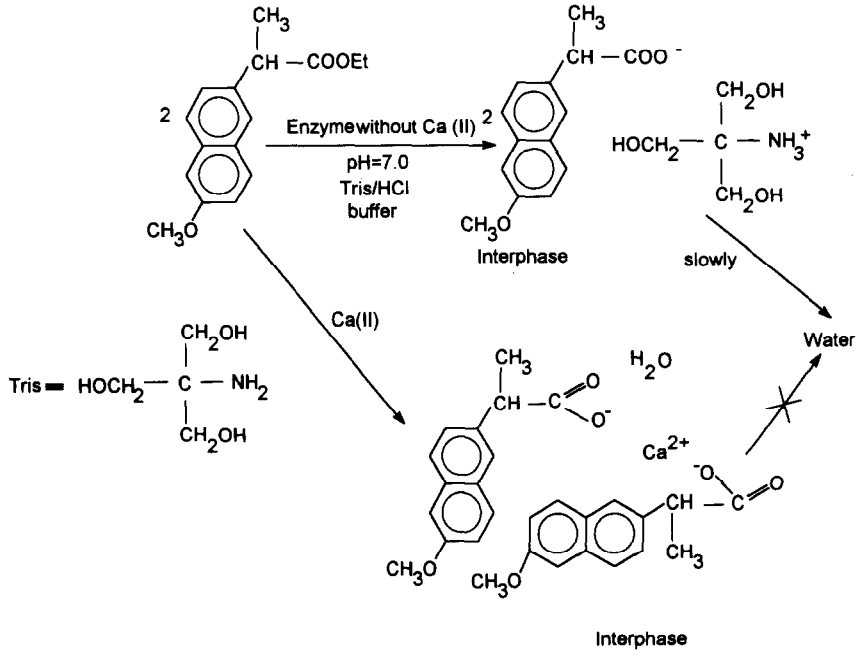
Fig. 3. Hydrolysis of the esters with LC (■), LB (*) and LBC (□) of *Candida cylindracea* (30°C, buffer Tris–HCl 0.1 M, pH = 7.0, time 96 h). (A)) (*R,S*)ethyl 2-phenylpropionate 0.1 M. (B)) (*R,S*)Ibuprofen ethyl ester 0.1 M. (C)) (*R,S*)Naproxen ethyl ester 0.02 M. (D)) (*R,S*)Ketoprofen ethyl ester 0.02 M.

We can observe that LB is more active than LC in all cases, as can be expected after the purification of the enzyme. In Fig. 3, we show that the influence of the purification grade is very important for the hydrolysis of ethyl (*R,S*)2-arylpropionates. In this case, LB is better biocatalyst than LS and LC (using the same conditions in all experiments).

Our results are in agreement with the results reported by Sih et al. [27] for the hydrolysis of 2-aryl- and 2-aryloxypropionates with crude lipase, and purified lipase using deoxycholate and organic solvents. These authors showed that there is an increase in k_{cat}/K_m ratio, after the purification steps, which seems to be related to a non-covalent modification of the enzyme. A similar hypothesis could be postulated in our case since only non-covalently bonded small molecules have been removed from the crude protein by our purification method (see experimental).

The presence of cations [(LBC), NaCl 0.125 M and $CaCl_2$ 0.125 M ($I=0.49$)] decreases the enzymatic activity of LB in the hydrolysis of the esters of (±)2-arylpropionic acids (Fig. 3). This effect is opposite to that observed in the case of the microemulsion of the olive oil (Fig. 1c). This finding must be explained by the different structure of fatty acid carboxylate and 2-arylpropionate. In the latter, the presence of aromatic rings both in the substrate and the product, could favour the stacking of the molecules on the interphase by π–π complexes. This effect cannot exist in the case of alkyl chain, where the steric hindrance would make difficult the organization of these molecules on the water/oil interphase. The presence of small ions could avoid the removal of ionic pairs [carboxylate (Tris–H^+)], from the interphase, giving stable stacked salts that will remain in the interphase favouring the inhibition of the enzyme by the reaction product. Therefore, the turnover number will decrease by inhibition by substrate. In the absence of small ions, Tris–H^+ cation (more lipophilic) could carry over in the microenvironment increasing the turnover number the carboxylate molecule (Scheme 4).

The greater the aromatic ring is the lower the productivity. We observe that 2-phenyl propionate and the ibuprofen ethyl ester give greater yields than naproxen and ketoprofen ethyl esters (Fig. 3). Indeed, diminishing the concentration of



Scheme 4.

naproxen and ketoprofen to the half of ibuprofen and 2-phenylpropionate ethyl ester, there is no enhancement of the hydrolysis of the first ones. This different behaviour cannot be related to the distance between the CO–OEt (attack point of Ser 209) and the end of the molecule (see Table 2 and Scheme 5). We can assume that the steric restriction of the ring or of ketoprofen to go into the lipidic tunnel [28] of the active site of the enzyme is the main reason for the low yields obtained with these molecules. Therefore, the lower percentage of (*S*)2-phenylpropionic acid achieved respect to (*S*)ibuprofen acid must be related to the lipidic properties of the substrate, which can be described by the Hansch parameter π. Both acids have the same aromatic ring. This parameter has been calculated from the literature [29] using 2-phenylpropionic acid as the reference compound. The obtained results are shown in Table 2 (Scheme 5).

In all cases the enzymatic hydrolysis was highly enantioselective but we observed an increase in the percentage of (*S*)acid obtained, at the same time, with LB compared to the native enzyme and LS. In Fig. 4 we show the ^{1}H-NMR spectra (CH_3–CH– zone) of the complex of (*R*,*R*) 1,2-diphenyl-1,2-diaminoethane with the 2-phenylpropionic acid produced in the hydrolysis of its ethyl ester with LB (Fig. 4A) (according to the procedure described by Fullwood [20]) and the ^{1}H-NMR spectrum of the racemic (±)2-phenyl propionic acid (Fig. 4B). Similar results are obtained in all cases. These results were confirmed by polarimetric measures. The specific rotatory power [α] agrees with the reported data for the pure *S*(+)isomer. As a consequence, it is evident that the native and the purified (LB) enzyme exhibited a marked enantioselectivity towards all the arylpropionic esters examined, but LB is more interesting as biocatalyst than LC and LS in this reaction, because it permitted greater yields than LC and LS with the same reaction time.

Table 2
Hydrolysis yield at 96 h of the 2-arylpropionic esters with LB and LC (% yield)

Substrate	π	Distance (A) [a]	% Hydrolysis	
			LB	LC
1	0.00	6.140	55.53	36.52
2	2.94	9.133	66.5	21.18
3	4.29	9.312	37.30	20.53
4	3.08	10.762	39.00	19.17

[a] Distance between the EtO–OC and the end of the aryl group.

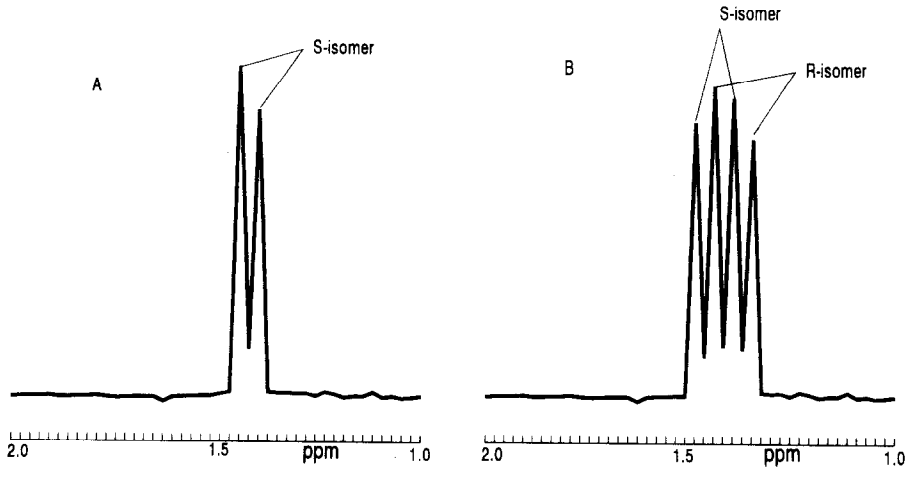
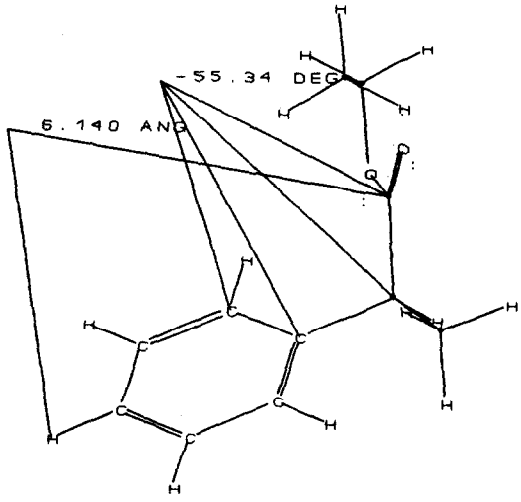


Fig. 4. ^{1}H-NMR of complex of (*R*,*R*)-2diphenylethane with 2-phenylpropionic acid obtained in the hydrolysis of the ethyl ester using LB (Fig. (A) and racemic 2-phenylpropionic acid (Fig. (B)).



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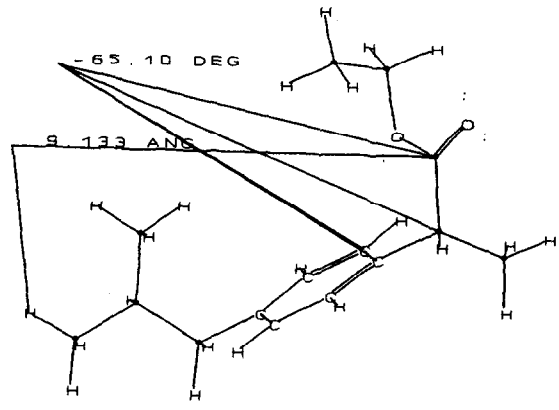

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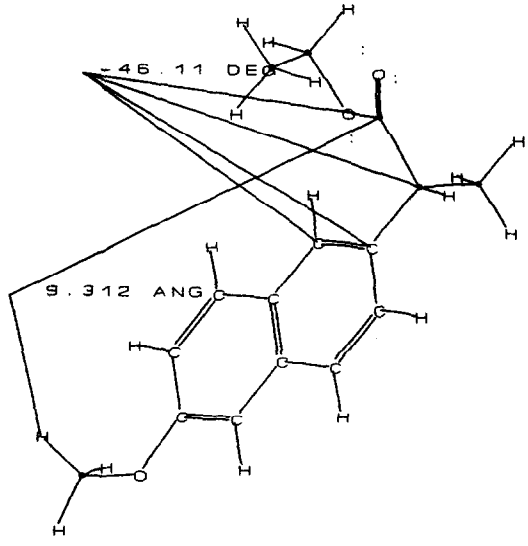

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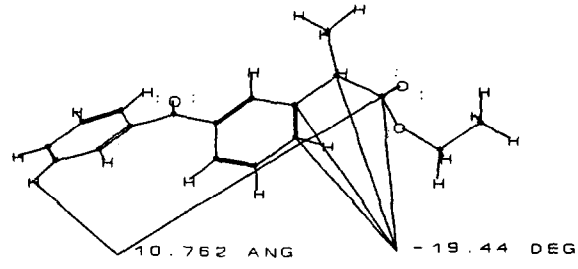

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Scheme 5.

Acknowledgements

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