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Lipases for biocatalysis: development of a chromatographic bioreactor

E. Calleri [a], C. Temporini [a], S. Furlanetto [b], F. Loiodice [c], G. Fracchiolla [c], G. Massolini [a,*]

[a] *Dipartimento di Chimica Farmaceutica, University of Pavia, Via Taramelli 12, 27100 Pavia, Italy*
[b] *Department of Pharmaceutical Sciences, University of Florence, Via G.Capponi 9, 50121 Florence, Italy*
[c] *Dipartimento Farmaco-Chimico, Via Orabona 4, 70126 Bari, Italy*

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Abstract

The development of a new chromatographic reactor based on immobilized *Candida rugosa* lipase (CRL) is described. The chromatographic system has been used to evaluate the rate differences by which the product enantiomers of esterolytic reactions catalyzed by immobilized CRL are obtained. The method has been applied to a series of racemic 2-aryloxyalkanoic acids and isosteric analogous methyl esters and to some non-steroidal antiinflammatory drugs 2-arylpropanoic acids methyl esters in order to study the structure effects on reaction rate and enantioselectivity. Lipase from *C. rugosa* has been non-covalently and covalently immobilized on HPLC chromatographic silica supports to develop an immobilized enzyme reactor (IMER). The reactor was connected through a switching valve to an analytical reversed-phase column, which was used for the on-line determination of the hydrolysis rate by peak area integration. The enantiomeric excess of the hydrolytic reaction products was determined off-line on a CSP utilizing immobilized penicillin G acylase (PGA-CSP).

Keywords: *Candida rugosa* lipases; Immobilized lipase; Enantioselective hydrolysis

1. Introduction

In the recent years, the use of biological systems for the production of optically enriched compounds has become an attractive alternative to classical methodologies of chemical asymmetric synthesis [1]. Different type of hydrolytic enzymes have been used so far for the preparation of pure enantiomers, among the enzymes used in organic chemistry, lipases play the most important role mainly because they accept a broad range of substrates while retaining high enantioselectivity [2–4].

Lipases are designed by nature to cleave carboxylic ester bonds in tri-, di-, and monoacylglycerols, however, lipases are also able to catalyze the reverse reaction (transesterification). These

* Corresponding author. Tel.: +39-0382-50-7383; fax: +39-0382-42-2975.
E-mail address: g.massolini@unipv.it (G. Massolini).

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enzymes are distributed among plants and animals and in additions many microorganisms are able to produce lipases. Lipases from different sources vary greatly in size and amino acid sequence; however, significant conservation of higher levels of protein structure was recorded, i.e. lipases possess very low sequence homology but very high three dimensional architectural similarity. All lipases possess a serine residue at the active site and such catalytically active amino acid is occluded by a polypeptide flap (or α-helical lid) that makes it inaccessible to substrate in aqueous media. Interfacial activation occurs when a lipase binds to a lipid interface via opening such flap; displacement of the lid exposes the catalytic site, converting the enzyme from the inactive to the active conformation [5,6].

The industrial versatility and unique catalytic performance of lipases have attracted increased interest in the biotechnology world and examples of their industrial applications are textile detergency, pharmaceutical synthesis, oils and fats bioconversions. Moreover, lipase-catalyzed reactions represent one of the best ways to produce enantiomerically pure compounds.

Among the commercially available lipases from different sources, *Candida rugosa* lipases (CRL) has been used in a wide range of catalytic reactions in both aqueous and water-restricted environments which include non-specific and stereo-specific hydrolysis. CRL has been exploited for the manufacture of fine chemicals, including pharmaceutical products and pure enantiomers. So far, no lipase is available with as broad a range of specificity as attributed to CRL.

CRL is mostly employed in the immobilized form; the use of such modified lipase is very advantageous since, beside a generally improved operational and temperature stability, it can be easily recovered at the end of the reaction and continuously recycled. Many different methods of immobilization have been applied including adsorption, cross-linking to multifunctional molecules (resins) and covalent attachment to different matrices [7–9]. The last immobilization technique is not as common as the physical adsorption, but presents the advantage of avoiding the desorption phenomenon. Nevertheless the main concern is to carry out the covalent attachment on amino acids that are not involved in the catalytic mechanism affecting the activity of the immobilized enzyme.

In spite of the great amount of work accomplished in biotechnology with immobilized lipases low attention has been paid to chromatographic reactors based on covalently bonded lipases [10,11]. The integration of enzyme reaction and product separation steps might be very interesting as the removal of the product of the enzyme reaction is a key factor in order to overcome product inhibition [12].

The objective of the present study was the development of a lipase-based analytical chromatographic stationary phase as useful tool to provide some new data on *C. rugosa* enantioselective esterolytic reactions, to estimate its selectivity quickly as well as to select suitable reaction conditions that are too often trial and error processes.

Different immobilization methodologies to produce an adequate CRL based stationary phase were considered. The properties of the most promising CRL-stationary phase were evaluated by using a series of racemic 2-aryloxyalkanoic acids and isosteric analogs methyl esters and some non-steroidal antiinflammatory drugs 2-arylpropanoic acids methyl esters. On purpose the enzymatically active reactor was coupled through a switching valve to an analytical column for the on-line separation and quantitation of the hydrolysis yield. The product enantiomeric excess was determined off-line on a PGA-chiral stationary phase [13,14].

2. Experimental

2.1. Reagents and materials

Lipase lyophilized powder from *C. rugosa* (CRL), *N*,*N*′-disuccinimidyl carbonate (DSC) and disuccinimidyl suberate (DSS) were purchased from Sigma-Aldrich (Milan, Italy). Potassium dihydrogen phosphate and dipotassium hydrogen phosphate used for the preparation of the mobile phases were of analytical grade and purchased from Merck (Darmstadt, Germany).

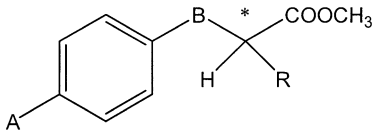
Aminopropyl silica packing material (Nucleosil-$3NH_2$, 5 μm particle size, 100 Å pore diameter) was obtained from Macherey-Nagel (Düren, Germany). Acetonitrile and 1-propanol were from Carlo Erba (Milan, Italy).

Water was deionized by passing through a Direct-Q™ system (Millipore, Bedford, MA, USA).

Racemic methyl esters 1–12, whose chemical structures are reported in Table 1, were prepared by treating the corresponding acids with a solution of diazomethane in diethyl ether. Mass and NMR spectra were consistent with the identity and purity of the so obtained compounds. Racemic acids and the dextro-isomers of the acids relating to methyl esters 1–11, were prepared according to our previous papers [15–17]. The enantiomeric elution order of the considered acid racemates on PGA-chiral stationary phase was determined in a previous paper [13].

Rac-ketoprofen was kindly donated by S.I.M.S s.r.l (Incisa Valdarno, FI, Italy) while *rac*-ibuprofen and *rac*-fenoprofen were purchased from Sigma (St. Louis, MO, USA). The methyl esters of ketoprofen, ibuprofen and fenoprofen were synthesized in our laboratory.

Table 1
Structures of a series of 12 structurally correlated 2-aryloxyalkanoic acid methyl esters and isosteric analogs



Racemate	A	B	R
1	Cl	O	CH_3
2	Cl	O	C_2H_5
3	Cl	O	C_6H_5
4	Cl	S	CH_3
5	Cl	S	C_2H_5
6	Cl	NH	CH_3
7	Cl	CH_2	CH_3
8	Cl	CH_2	C_2H_5
9	Br	O	CH_3
10	F	O	CH_3
11	CH_3	O	CH_3
12	COC_6H_5	O	CH_3

2.2. Equipment

Chromatographic experiments were performed with three HPLC systems. One system (System A) was an Hewlett Packard HP 1050 liquid chromatograph (Palo Alto, CA, USA) with a Rheodyne sample valve (20-μl loop) equipped with an Hewlett Packard HP 1050 variable-wavelength detector and the enzyme reactor. The mobile phase consisted of phosphate buffer (pH 7.0; 50 mM) and the flow rate was set at 0.5 ml/min.

The second equipment (System B) consisted of an Hewlett Packard HP 1100 liquid chromatograph with a Rheodyne sample valve (20-μl loop) equipped with an Hewlett Packard HP 1100 variable-wavelength detector, a HP 1100 thermostat and a Zorbax Rx-C8 (5 μm, 15 × 4.6 mm I.D.) column (Agilent Technologies, Waldbronn, Germany). The mobile phase was phosphate buffer (pH 7.0; 50 mM)–methanol. The analyses on system B were carried out applying the following mobile-phase gradient: phosphate buffer (pH 7.0; 50 mM)–methanol (60:40, v/v) for the first 10 min, step gradient to phosphate buffer (pH 7.0; 50 mM)–methanol (40:60, v/v) at 10.01 min and return to the starting condition at 25 min. The flow rate was 1.0 ml/min and the detection was performed at 225 nm.

Systems A and B could be used independently or the eluent from System A could be directed onto System B through a HP six-port switching valve as outlined in Fig. 1. Both systems were connected to a HPLC ChemStation (Revision A.04.01).

System C consisted of a Hewlett Packard HP 1100 liquid chromatograph with a Rheodyne sample valve (20-μl loop) equipped with a Hewlett Packard HP 1100 diode-array detector, a HP 1100 thermostat and a PGA-epoxide chiral stationary phase (150 × 4.6 mm I.D.).

The mobile phase was phosphate buffer (pH 7.0; 50 mM), the flow-rate was maintained at 0.8 ml/min and the detection was performed at 225 nm.

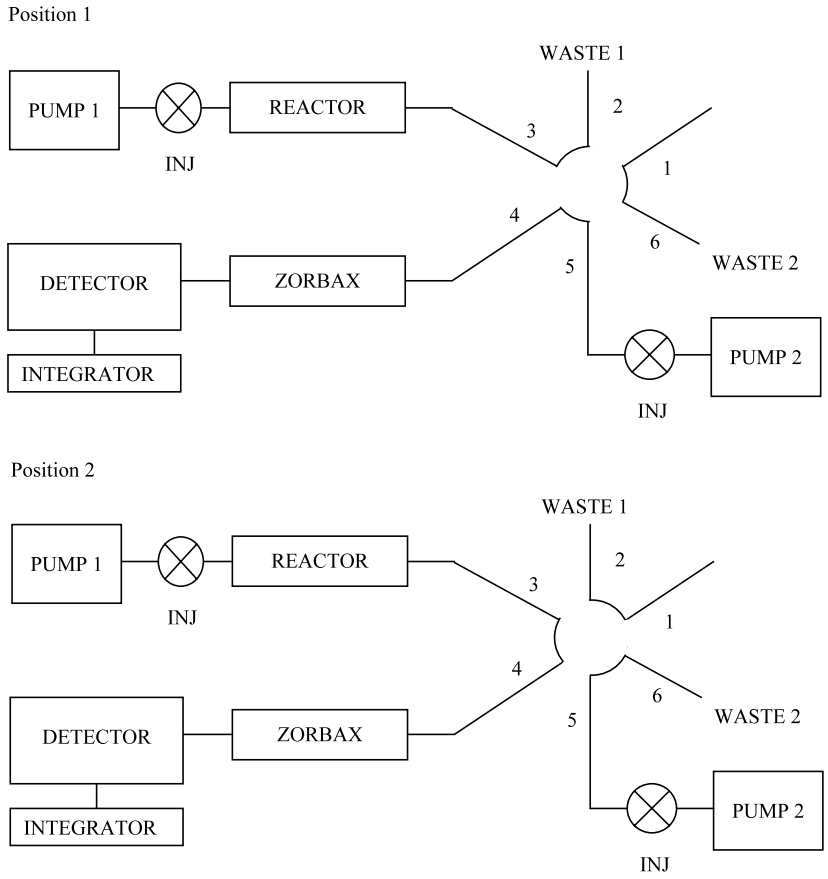


Fig. 1. Chromatographic system coupling the IMER with the reversed-phase analytical column. The substrate is loaded onto the IMER using Position 1; the product and the unreacted substrate are switched to the analytical column using Position 2; the conversion percentage is measured on the analytical column using Position 1.

2.3. Immobilization techniques

The in situ process, which consists of the attachment of proteins directly in pre-packed columns, was used to immobilize lipase to create an immobilized enzyme reactor (IMER) [18]. The physical adsorption of CRL was carried out on a RP18 stationary phase by hydrophobic interactions while the covalent immobilization of lipase was carried out by using DSC and DSS activating agents on aminopropyl-silica packed into stainless-steel columns (5 μm, 50 × 4.6 mm I.D.) by conventional high-pressure slurry-packing procedures. The reaction schemes for the covalent immobilization of lipase are reported in Fig. 2.

2.3.1. Lipase immobilization via physical adsorption

The immobilization was carried out at a pH value close to the isoelectric point (about 5). At room temperature an Hypersil-ODS stationary phase (50 × 4.6 mm I.D.) was connected to System A and an enzyme solution [200 mg CRL dissolved in 100 ml of phosphate buffer (pH 5.0; 1 mM)] was recycled through the column at a flow-rate of 0.5 ml/min for 24 h. The amount of immobilized lipase

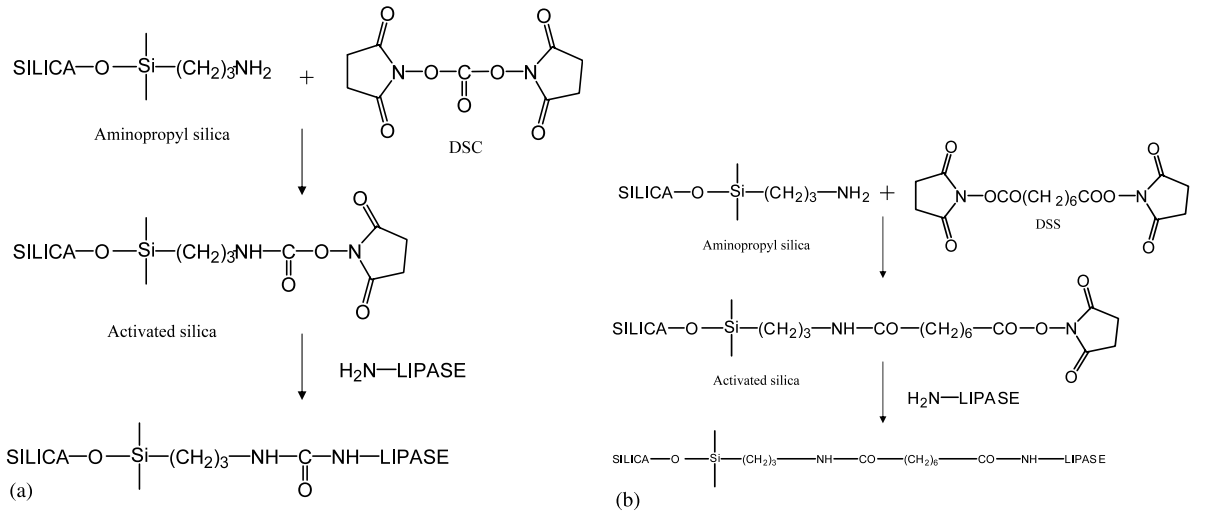


Fig. 2. Scheme for the covalent immobilization of lipase. (a) Lipase immobilization on aminopropyl silica activated with DSC. (b) Lipase immobilization on aminopropyl silica activated with DSS.

was determined by the UV absorbance decrease of the enzyme solution before and after the immobilization procedure and was found to be 143.94 mg.

2.3.2. Covalent immobilization of lipase on DSC-activated aminopropyl silica

2.25 g of DSC were dissolved in 100 ml acetonitrile and the resulting solution was continuously circulated for 18 h at 0.5 ml/min through the column previously equilibrated with the same solvent. The column was then washed at the same flow rate first with 60 ml acetonitrile and then with 60 ml of phosphate buffer (pH 7.0; 1 mM). 200 mg lipase were dissolved in 100 ml of phosphate buffer (pH 7.0; 1 mM) and the enzyme solution continuously circulated at 0.3 ml/min. After 24 h the column was washed with 200 ml of phosphate buffer (pH 7.0; 20 mM), 200 ml of a solution of NaCl (0.5 M) and finally with 100 ml of a glycine solution (0.2 M) to block the remaining activated groups.

2.3.3. Covalent immobilization of lipase on DSS-activated aminopropyl silica

The immobilization on aminopropyl-silica column activated with DSS was carried out as follows.

The column was first washed (1 h for each eluent at flow rate 0.5 ml/min) with acetonitrile and with $NaHCO_3$ (0.1 M)–CH_3CN (67:33, v/v). Then the stationary phase was activated by recycling 0.450 g DSS in 30 ml acetonitrile for 12 h followed by washing with 15 ml $NaHCO_3$ (0.1 M) at 0.5 ml/min, 20 ml of water–acetonitrile (33:67, v/v) and finally with 100 ml of $NaHCO_3$ (0.1 M). A solution of 96 mg lipase in 40 ml of a $NaHCO_3$ solution (0.1 M) was recirculated through the column at a flow rate of 0.5 ml/min for 16 h, flushing and back flushing every 15 min during the first hour, every 30 min during the following 3 h.

After the immobilization procedure the column was rinsed for 1 h with phosphate buffer (pH 7.0; 50 mM) at flow rate of 0.5 ml/min. After that, the column was flushed with 50 ml of a glycine solution (1 M) in phosphate buffer (pH 7.0; 50 mM) and then rinsed with the same phosphate buffer.

2.4. Immobilization yield and activity determination

The amount of immobilized enzyme on the activated DSC and DSS aminopropyl silica columns, as determined by elemental analysis, was

found to be 67.54 and 82.79 mg/g solid support, respectively.

2.5. Sample preparation

A known amount of racemic substrate was dissolved in 1-propanol and the resulting solution was diluted with the mobile phase buffer to a final concentration of 1 mM.

2.6. Calculations

The enantioselectivity, E, for an irreversible enantiomer-differentiating enzyme-catalyzed hydrolysis is expressed as:

$$E = \ln[1 - C(1 + ee_P)]/\ln[1 - C(1 - ee_P)] \quad (1)$$

where C is the degree of substrate conversion, or conversion factor.

The product enantiomeric excess was calculated as follows:

$$ee_P = (x_1 - x_2)/(x_1 + x)_2 \quad (2)$$

where x_1 and x_2 denote the concentrations of the two acidic enantiomers separated on the chiral stationary phase.

2.7. Analytical procedure

Quantification of the conversion degree and of the enantiomeric excess for the calculation of enantioselectivity was carried out using the following chromatographic set-up.

1 mM substrate solutions were injected onto System A at valve position 1 (see Fig. 1). Loading eluent was delivered by the System A pump at a flow-rate of 0.5 ml/min. After 0.1 min the valve had been switched to position 2 and the analytes (products and unreacted substrate) were flushed and focused for 10 min directly to the reversed-phase analytical column. The valve was then switched back to its original position for separation with eluent delivered by System B. A representative chromatogram showing the separation of the product from the unreacted substrate racemate 9 is given in Fig. 3a.

The rates of substrate conversions (C) were calculated from the peak areas of the remaining ester building for each analytes calibration curves between 0.5 and 100% conversion.

The product of the enzymatic hydrolysis was collected from the analytical column and directly injected off-line onto system C for chiral resolution and determination of the product enantiomeric excess (ee_P) on a PGA-CSP. A representative chromatogram showing the separation of the (S)- and (R)- product acidic enantiomers of racemate 9 is reported in Fig. 3b.

3. Results and discussion

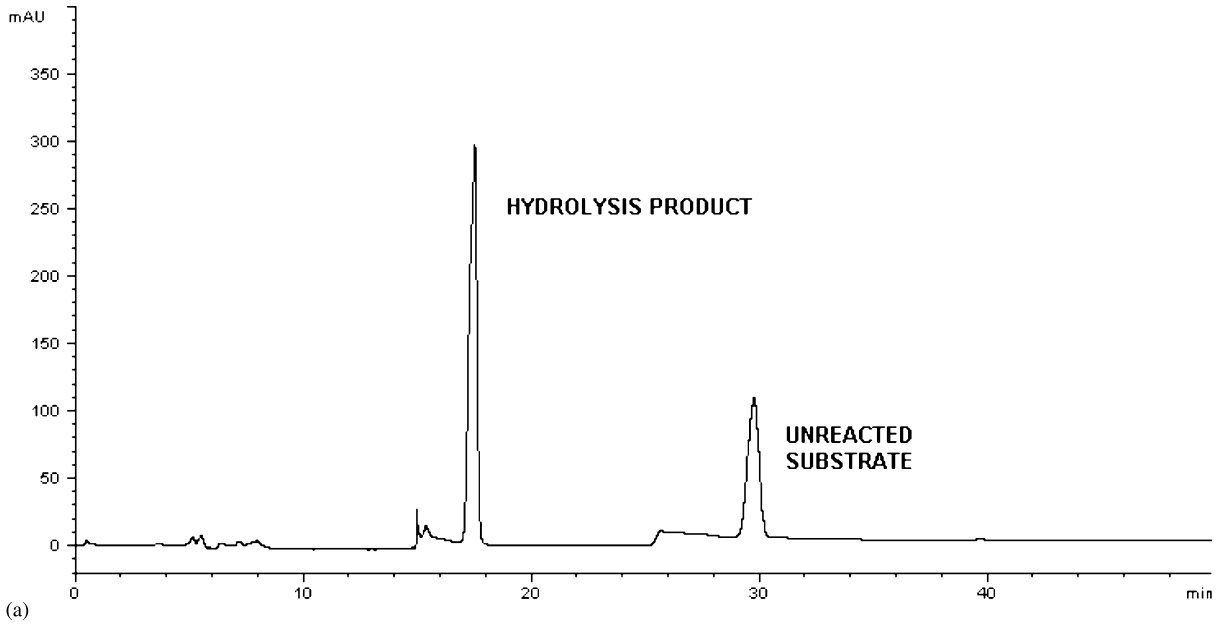
3.1. Lipase immobilization

The main problem in developing the chromatographic bioreactor was related to the catalytic mechanism of this enzyme which require an interfacial activation. This capability is due to the unique structural characteristic of lipases: these latter indeed contain an α helical oligopeptide unit that covers the entrance to the active site. This so-called lid only moves upon access to a hydrophobic interface. For this reason, at the beginning of this study the physical adsorption of lipase on a RP18 stationary phase was considered. This procedure was based on the assumption that the large hydrophobic area surrounding the active site of lipases is the one mainly involved in their adsorption on strongly hydrophobic solid surfaces. This immobilization method should be able to promote an hyper-activation of the enzyme increasing its activity. The yield of immobilization was very high but unfortunately the chromatographic reactor was unstable due to the weak enzyme-support interactions that lead to enzyme bleeding and unreproducible chromatographic results (data not shown). Moreover, the addition of methanol to the mobile phase to decrease the analyte retention times caused the complete desorption of the enzyme from the hydrophobic support.

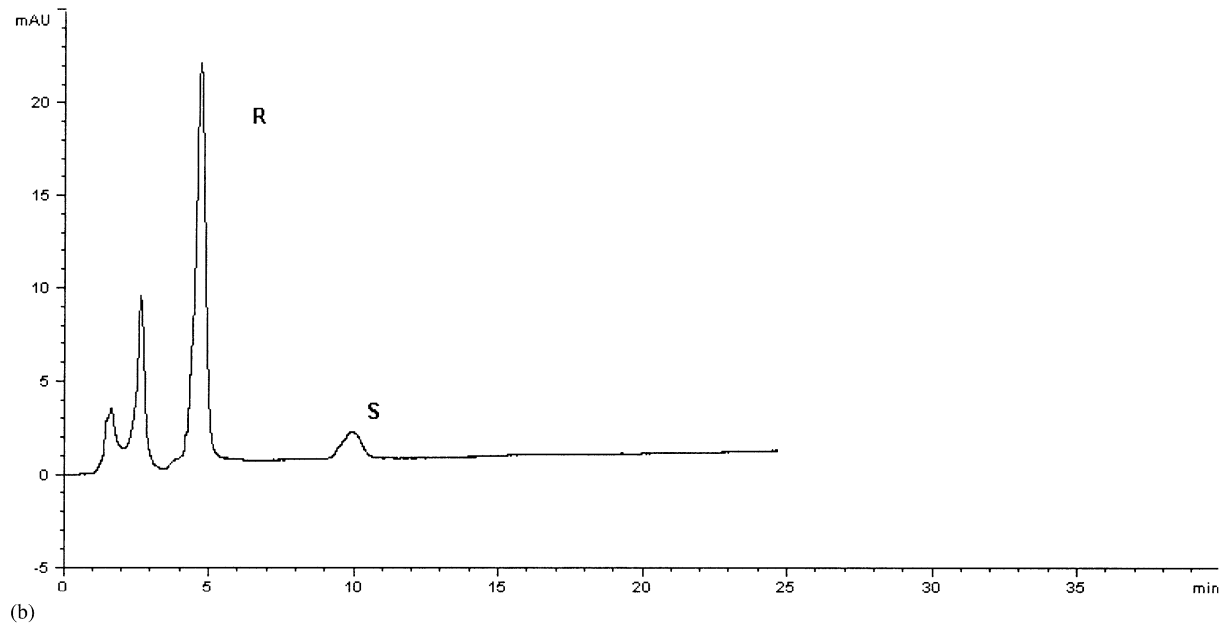
To avoid the desorption phenomenon and to increase the operational stability we moved to the covalent immobilization. Lipases were first immobilized on aminopropyl silica activated with DSC.

The hydrolysis of ketoprofen methyl ester was used as a standard assay to test the on-line activity

of the covalent immobilized enzyme. A 1 mM solution of ketoprofen methyl ester was injected onto the enzymatic column flushed with 50 mM phosphate buffer pH 7.0 at 0.5 ml/min. Only one peak appeared on the chromatogram. The fraction corresponding to the eluted peak was switched



(a)



(b)

Fig. 3. (a) Chromatogram showing the separation of the product from the unreacted substrate racemate 9 obtained on-line with the coupled IMER–RP system; see text for experimental conditions. (b) Chromatogram showing the separation of the (S)- and (R)-product acidic enantiomers of racemate 9 on PGA-epoxide chiral stationary phase (150 × 4.6 mm I.D.). See text for chromatographic conditions.

onto the reverse-phase column for peak identification and determination of the conversion degree, an 1.15% hydrolysis was reached.

As previously reported, interfacial activation occurs when lipase binds to a lipidic interface by opening the α-helical lid. Aminopropyl silica has C3 chains which are very weakly hydrophobic and for this reason, longer carbon chains were introduced onto the aminopropyl support to increase the lipophilicity of the support. *N*,*N*′-disuccinimydilsuberate (DSS) has C6 chains and should be able to play an hydrophobic role. The effect of extending the carbon chain of the hydrophobic arm was quite surprising: the hydrolysis rate slightly increased (6.7%) but the chromatographic profile presented two peaks, that once identified, corresponded to the acidic product and to the unhydrolized ester.

The developed chromatographic support was able to separate the product from the unreacted substrate, additional interactions with the hydrophobic spacer might contribute to the chromatographic separation of the product from the unreacted substrate, however, not chromatographic enantio-discrimination was observed (Fig. 4). The integration of enzyme reaction and product separation steps is important because the removal of the product of the enzyme reaction is a key factor especially when accumulation of product inhibits enzyme activity.

3.2. Enantioselectivity and reaction rate

The hydrolysis of the racemates of some 2-aryloxyalkanoic acids methyl esters was considered. In this investigation we evaluated the overall rate differences produced by changes in the substrate structure on enantioselectivity.

In order to quantitatively determine the total ester hydrolysis of different substrates, the enzymatically active reactor was coupled through a switching valve to an analytical column. The column-switching system was used to calculate the rate of substrate conversion (C) and to collect the product whose enantiomeric excess (ee_P) was calculated off-line for the determination of enantioselectivity (E). The C and ee_P values together with E, calculated from the chromatographic data are reported in Table 2. All transformations proved to be highly enantioselective in favor to the (R)-form with exception of compound 7 for which the fast reacted enantiomer is (S)-form as a

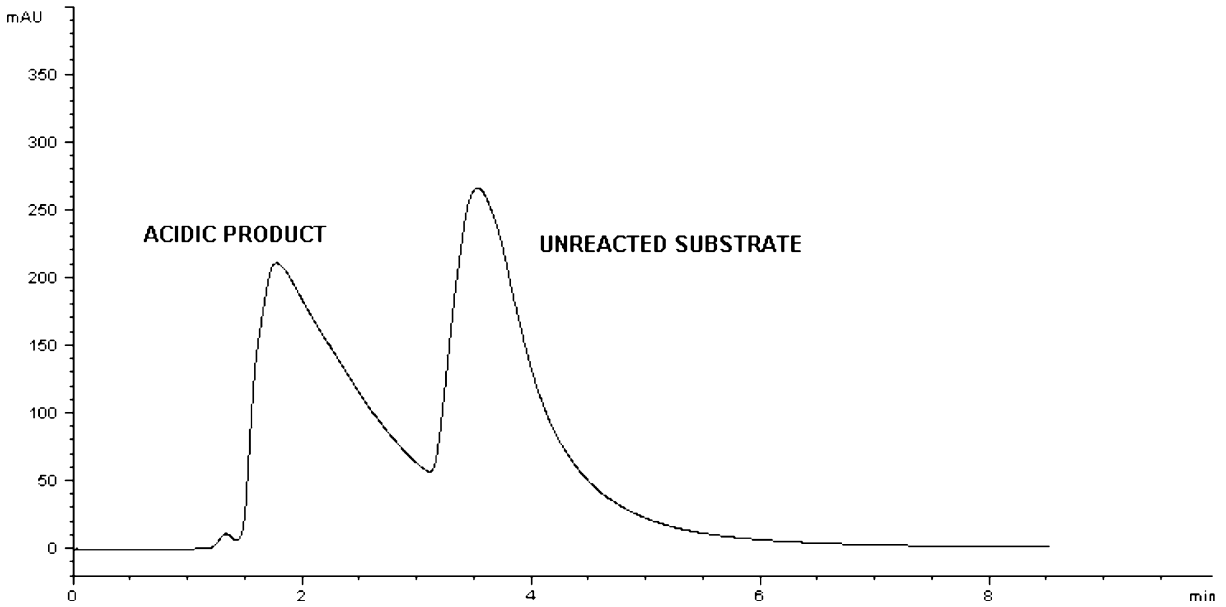


Fig. 4. Chromatogram of racemate 9 on the IMER. Chromatographic conditions in the text.

Table 2
Relative initial rates, enantiomeric excess and enantioselectivity for the lipase-catalyzed hydrolysis of the esters investigated

Substrate	C (%)[a]	ee_P (%)[b]	F.r.[c]	E[d]
1	48.41	71.44	(R)	11.0
2	23.00	19.68	(R)	1.6
3	6.60	n.d.	n.d.	n.d.
4	33.15	41.62	(R)	3.0
5	4.17	n.d.	n.d.	n.d.
6	4.53	n.d.	n.d.	n.d.
7	33.66	68.87	(S)	7.7
8	1.06	n.d.	n.d.	n.d.
9	54.51	84.36	(R)	33.0
10	27.65	n.d.	n.d.	n.d.
11	53.04	81.86	(R)	25.0
12	43.78	80.39	(R)	17.0
Ketoprofen	6.77	n.d.	n.d.	n.d.
Ibuprofen	6.80	n.d.	n.d.	n.d.
Fenoprofen	27.33	n.d.	n.d.	n.d.

Experimental conditions: 50 mM phosphate buffer, pH 7.0, temperature = 25 °C, flow 0.5 ml/min. n.d., not determined (ee_p was not determined for substrate conversion < 10%).

[a] C = degree of substrate conversion (%) experimental error ±2.1%.

[b] ee_P, Enantiomeric excess (%).

[c] F.r., fast reacted enantiomer.

[d] E., enantioselectivity.

result of the change in priority of the Cahn, Ingold, Prelog descriptor system for chiral molecules. Clearly the rates of hydrolyses are strongly dependent on the substrate structures as small structural modifications, changed dramatically the conversion rate and/or the enantiospecificity of the reaction involving the selected substrates.

An increase of the dimension of the α-substituent (compare compound 1 with 2 and 3, 4 with 5 and 7 with 8) lead to a strong reduction of the conversion rate and enantioselectivity, this trend leads us to assume the presence of steric hindrance in the active site. The substituent on the aromatic ring (compounds 1, 9–12) does not influence significantly the reaction rate and enantioselectivity with the only exception of compound 10 with a fluorine atom in the para-position. The low degree of substrate conversion and enantiomeric excess can be ascribed to the strong inductive electron withdrawing effect of this substituent; on the contrary when considering the isosteric substitution in α-position (compounds 1,4,6,7) the lower conversion rate and enantioselectivity were observed for compound 6 with a NH group close to the stereogenic center. Probably, this group, acting as a hydrogen bond donor, makes possible an additional interaction with the lipase which results unfavorable to hydrolysis and chiral discrimination processes. Notably, when an ethyl is present on the stereogenic center in place of the methyl group, the conversion rate, though lower, shows a more strict dependence on the isosteric substitution (compare compound 2 with 5 and 8).

4. Conclusion

This research has brought forth an understanding of how the immobilization process can influence the performances of a lipase-based chromatographic reactor. The hydrolytic activity increased by increasing the hydrophobic arm and moreover the chromatographic separation of the product from the unreacted substrate was reached.

In this study the substrate specificity of lipase from *C. rugosa* towards a series of structurally related racemic 2-aryloxyalkanoic acids methyl esters was investigated showing that the immobilized enzyme displays high R-enantiopreference. The rates of hydrolyses were strongly dependent on the substrate structures.

The described system can be successfully used to rapidly screen the stereoselective activity of lipases towards different substrates and for the systematic analysis of the experimental variables that control a highly stereoselective resolution including solvent, temperature and ionic strength.

In addition, after E-values suitable for practical purposes have been reached, the described integration of enzyme reaction and product separation steps might be exploited in the preparation of significant quantities of the desired enantiomer.

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