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Influence of –OR ester group length on the catalytic activity and enantioselectivity of free lipase and immobilized in membrane used for the kinetic resolution of naproxen esters

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Abstract

Lipases are suitable catalysts for the kinetic resolution of racemic mixtures due to their ability to discriminate between enantiomers. For this reason, they have been studied largely to develop reactors for the production of optically pure enantiomers. The main problem in these productive systems is enzyme stability and enantiocatalytic selectivity as a function of time. In this work, the enantiocatalytic properties of lipase as a function of the –OR group length were studied. The methyl, butyl, and octyl esters of naproxen were synthesized and used as reagents. The lipase was used as a free agent and immobilized in a polymeric membrane reactor. The results show that selectivity and stability of the enzyme improved while catalytic activity decreased with the –OR length group. The immobilized enzyme had higher activity compared with the free enzyme.

Keywords: Lipase; Immobilization; Enantioselectivity; –OR group ester length; Kinetic resolution; Membrane reactor

1. Introduction

Lipases have attracted considerable attention as biocatalysts for hydrolytic and esterification reactions [1]. In particular, due to their ability to catalyze conversions of low water-soluble substrates at the oil–water interface and to discriminate between enantiomers, they have been used as phase-transfer catalysts for enantioselective bioconversion in the production of optically pure enantiomers [2]. The need to use pure enantiomers for the preparation of drugs, foods, agrochemicals, and so on is well recognized, because of the well-known different recognition that enantiomers may have at the molecular level [3]. Often, only one of the two isomers has a beneficial effect, whereas the other may have side effects; even when the unwanted isomer is not harmful, it represents a useless load to be metabolized by the liver.

Kinetic resolution by means of enantioselective catalysts is a suitable method for obtaining pure enantiomers, especially when combined with separation systems, such as membrane operations, able to separate the produced enantiomer from the racemic substrate. The combination of the enantioselective properties of the catalyst and the separative properties of the membrane allows the conversion and separation of the enantiomer in a single step.

Lipases have been used for the development of enzyme membrane reactors able to carry out reaction and product separation [4]. The main problem in using enzymes on a productive scale is their stability when used as free agents. For this reason, their stability is improved by heterogeneization with a solid support, such as a polymeric or inorganic membrane. On the other hand, immobilized enzymes can experience a reduction in their native catalytic properties. Although this is not a general rule [5], an immobilized enzyme is often seen to increase stability while decreasing catalytic activity [6,7].

In this work, the possibility of identifying substrates in which the lipase would maintain high selectivity and stability as either a free enzyme or an immobilized enzyme is investi-

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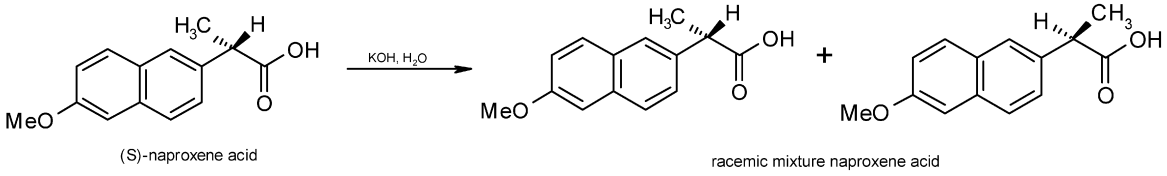
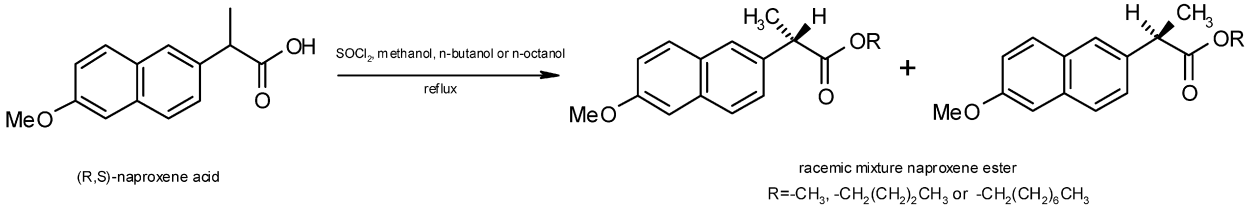


Scheme 1. Racemization of (*S*)-naproxen acid.



Scheme 2. Esterification of (*R*,*S*)-naproxen acid.

gated. In particular, the influence of esters with different –OR length groups on lipase enantioselectivity and activity is studied. The hypothesis that the length of the ester groups could affect the enantioselectivity arose out of the observations that (i) the lipase natural substrate, oils and fats, are esters of glycerol with carboxylic acids having a long chain, usually 12 or more carbon atoms; (ii) in ester synthesis, lipases prefer short-chain fatty acids and alcohols [1]; and (iii) in racemate ester hydrolysis, the degree of enantioselectivity varies according to the size of the substituents at the stereocenter and the pH, which affects the active site conformation [8,9].

The results indicate that the catalytic activity of lipase decreased and the enantioselectivity increased with –OR ester group length. The observed activity of the immobilized enzyme for the three esters proved higher than that for the free enzyme.

2. Materials and methods

2.1. Chemicals

Lipase from *Candida rugosa* (CRL) type VII was purchased from Sigma (E.C.3.1.1.3). The lot contained 1 g of protein per 5.88 g of raw powder and 819 units mg^{-1} protein. The enzyme solution was prepared in a 50 mM phosphate buffer (pH 7.00), then centrifuged at 5000 rpm for 15 min before use to remove the suspended solids. It was verified that the concentration of protein and the enzyme activity did not change after centrifugation.

The substrates were different esters of (*R*,*S*)-naproxen: methyl ester, butyl ester, and octyl ester. They were synthesized with the following reagent: (*S*)-naproxen acid, dry thionyl chloride, methanol, *n*-butanol (99+%), *n*-octanol (99+%), dichloromethane, chloride acid (6 M) from Sigma–Aldrich; sodium sulfate anhydrous (99%) from Fluka; KOH from Carlo Erba; and silica gel 60(70–230) from Merck. During enzymatic hydrolysis, these substrates were dissolved in isooctane. Along with the synthesized esters, the initial catalytic properties of the lipase were also evaluated using its natural substrate, triglycerides present in olive oil. Asymmetric membranes made of polyamide with a nominal molecular weight cutoff of 50 kDa were kindly provided by Berghof (Germany).

2.2. Synthesis of (*R*,*S*)-naproxen esters

The racemic esters were prepared in the laboratory according to the classical procedure, starting from the commercially available (*S*)-naproxen acid. Therefore, performing racemization before the esterification was necessary.

2.2.1. Racemization of (*S*)-naproxen acid

A 1-g sample of (*S*)-naproxen acid was dissolved in a phial with a screw cap containing 30 ml of KOH (2 M) solution (Scheme 1). The solution was refluxed for 14 h at 150 °C. The reaction mixture was cooled at room temperature, and an aqueous solution of HCl (6 M) was added. (*R*,*S*)-naproxen was precipitated as a white powder and washed with distilled water. The precipitate was oven-dried, and the racemization was verified by high-performance liquid chromatography (HPLC) analysis.

2.2.2. Esterification of (*R*,*S*)-naproxen acid

Racemic naproxen methyl ester, butyl ester, and octyl ester were synthesized by the same general esterification method using thionyl chloride and methanol, *n*-butanol, or *n*-octanol anhydrous. Thionyl chloride ($SOCl_2$) was added dropwise to a cooled, stirred suspension of (*R*,*S*)-naproxen acid in alcohol. The reaction mixture was refluxed for 2 h (Scheme 2). Then the solvent was evaporated, and the residue was purified by column chromatography using SiO_2 as an adsorbent and dichloromethane as a solvent. The residual methanol, butanol, or octanol was removed by vacuum evaporation. After drying, naproxen ester powder was obtained. The conditions for the ester preparation and the related yields are summarized in Table 1. The esterifications were verified by IR, GC/MS, and ^{1}H NMR.[1]

[1] *Methyl esters of (R,S)-naproxen*; IR (KBr) ν (cm^{-1}): 3143, 2975, 1739, 1605. GC/MS: 244 (46%), 185 (100%). ^{1}H NMR ($CDCl_3$) δ (ppm): 1.60

Table 1
Amounts of components used in the production of racemic methyl, butyl, and octyl esters and respective yields

Ester	(*R*,*S*)-naproxen acid		Alcohol		Thionyl chloride, $SOCl_2$ (mol)	$SOCl_2$ (ml)	Yield (%)
	(ml)	(g)	(mol)	(ml)			
Methyl ester	1.7×10^{-2}	4	9.8×10^{-1} (methyl alcohol)	40	2.9×10^{-2}	2.1	73
Butyl ester	3.0×10^{-3}	6.9×10^{-1}	1.1×10^{-1} (butyl alcohol)	18	5.0×10^{-3}	4.0×10^{-1}	75
Octyl ester	5.7×10^{-3}	1.32	2.0×10^{-1} (octyl alcohol)	32	9.5×10^{-3}	7.0×10^{-1}	70

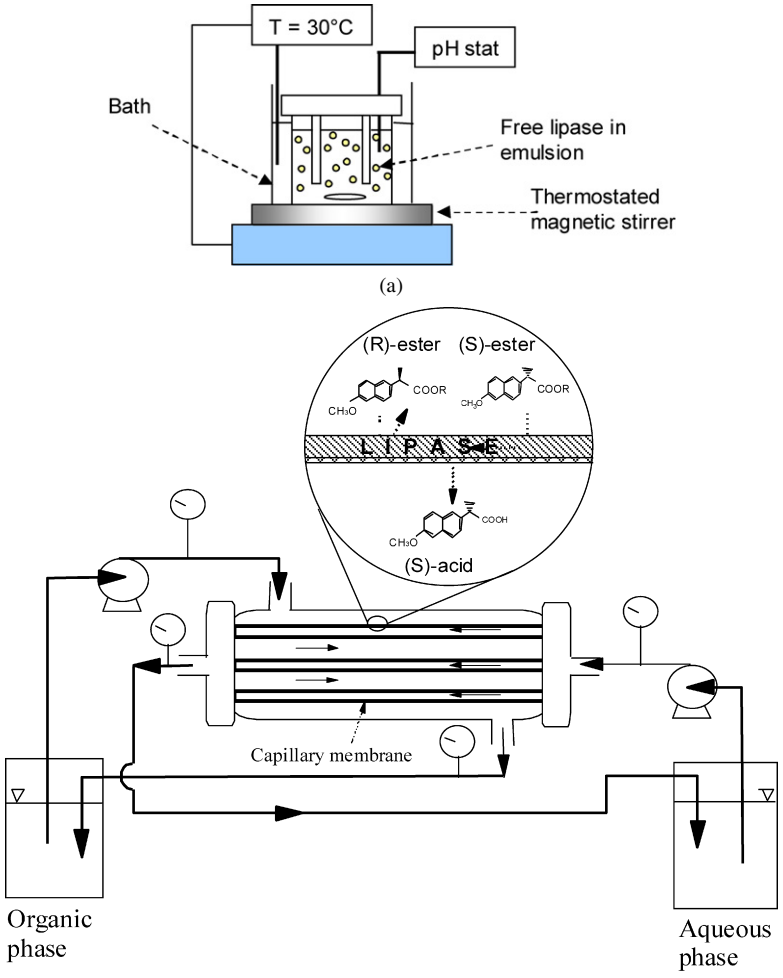


Fig. 1. Schematic representation of equipment used for testing the catalytic properties of free and immobilized lipase, respectively: (a) stirred tank reactor and (b) two-separate-phase enzyme membrane reactor.

(d, 3H, $J = 7.1$ Hz), 3.69 (s, 3H), 3.89 (q, 1H, $J = 7.1$ Hz), 3.93 (s, 3H), 7.16 (m, 2H), 7.43 (dd, 1H, $J = 8.5$ Hz), 7.71 (m, 3H).

Butyl esters of (R,S)-naproxen; IR (KBr) ν (cm^{-1}): 3055, 2954, 1730, 1606. GC/MS: 286 (45%), 185 (100%). ^1H NMR ($CDCl_3$) δ (ppm): 0.88 (t, 3H, $J =$ 7.4 Hz), 1.31 (sext, 2H, $J = 7.5$ Hz), 1.54 (d, 3H, $J = 7.0$ Hz), 1.61 (m, 2H), 3.88 (q, 1H, $J = 7.1$ Hz), 3.93 (s, 3H), 4.10 (m, 2H), 7.15 (m, 2H), 7.43 (dd, 1H, $J = 8.4$ Hz), 7.71 (m, 3H).

Octyl esters of (R,S)-naproxen; IR (KBr) ν (cm^{-1}): 3100, 2927, 1733, 1606. GC/MS: 342 (55%), 185 (100%). ^1H NMR ($CDCl_3$) δ (ppm): 0.86–1.61 (m, 18H), 3.86 (q, 1H, $J = 7.1$ Hz), 3.91 (s, 3H), 4.09 (t, 2H, $J = 6.6$ Hz), 7.14 (m, 2H), 7.43 (dd, 1H, $J = 8.4$ Hz), 7.71 (m, 3H).

2.3. Equipment for lipase performance measurements

Two reactor systems were used to evaluate the catalytic performance and the selectivity of the lipase regarding the kinetic resolution of the naproxen esters. In one case, a stirred tank reactor was used to test the lipase freely suspended in an oil-in-water emulsion. The reactor comprised a 50-ml tank containing baffles to avoid a vortex while dispersing the two immiscible phases into each other (Fig. 1a). The tank was immersed in a thermostatic bath, and operating conditions (e.g., temperature,

pH, stirring rate) were monitored and controlled during the experiments.

When an immobilized enzyme was used, a two-separate-phase membrane reactor was applied (Fig. 1b). The system comprised an enzyme-loaded membrane module containing asymmetric membranes made of polyamide. The enzyme was immobilized by cross-flow ultrafiltration, and the amount of enzyme entrapped within the membrane was determined based on the mass balance between the initial and final solutions. The organic phase contained the substrate and was recirculated along the shell side while the aqueous phase extracted the product and was recirculated along the lumen side. The two phases were contacted and kept separated at the membrane level. The reaction was monitored by measuring the product extracted into the aqueous phase. In this case, observed properties instead of intrinsic ones were considered, because transport phenomena through the membrane to the bulk phase also occur. The data representation neglected transport phenomena, because the reaction rate and mass transfer rate measurements demonstrated that the immobilized system still worked in limited reaction conditions. Therefore, only some delay in the reaction rate was observed, but the catalytic performance was not affected by the mass transfer properties.

2.3.1. Lipase activity measurements

The free lipase was tested in the stirred tank reactor. The pH and temperature were maintained constant at 7.00 and 30 °C. When olive oil was used as a substrate, the reactions were monitored online by titration with NaOH 20 mM using a Mettler DL25 automatic titrator. The reaction mixture was formed by 19 ml of phosphate buffer 50 mM, 1 ml of olive oil, and 2 ml of enzyme solution 3 $g_{\text{raw powder}}/L$. The mixture was stirred at 500 rpm with a magnetic stirrer to disperse the two immiscible phases and create the oil–water reaction interface. When synthetic esters were used, the reactions were monitored offline by taking samples at given intervals of time and then measuring (*S*)- and (*R*)-naproxen acid by HPLC.

The reaction mixture comprised 23 ml of phosphate buffer and 20 ml of substrate solution composed of naproxen ester 5 mM in isooctane and 3 ml of enzyme solution 2 $g_{\text{raw powder}}/L$ stirred at 500 rpm.

During the reaction, a 3-ml sample was taken from the aqueous phase reaction volume and replaced with 3 ml of a similar enzyme solution (0.23 $mg_{\text{raw powder}}/ml$) so as to not have a negative effect on the reaction due to enzyme mass variation. The samples were filtered with a 50-kDa membrane to stop the reaction and to purify the sample for HPLC analysis.

The concentration of the chiral product was measured using a chirobiotic V (250 × 4.6 mm) column protected by the precolumn chirobiotic V guard (from Astec). The mobile phase was a mixture of THF and an aqueous solution (pH 7) containing 0.1% of TEA (10:90 v/v). The analyses were performed at a flow rate of 1 ml/min and a wavelength of 254 nm. The (*S*)-naproxen acid was eluted faster than the (*R*)-naproxen acid, with retention times of 6.5 and 7.5 min, respectively. The concentration of the samples was evaluated using the external standard method. The error associated with the analyses, estimated as the ratio between the standard deviation and the average value over eight injections of the same sample, was 0.9%.

The total mass of the reaction product at time t was calculated as follows:

$$\text{mg}_{\text{tot}(t)} = C_t V_{\text{tot}} + \sum C_n V_n. \tag{1}$$

Here C_t = concentration at time t; V_{tot} = total aqueous reaction volume at time t; C_n = concentration of a sample; V_n = volume of a sample.

The product mass as a function of time allow calculation of the total conversion, c, the enantiomeric excess, ee, and the enantioselectivity, E,

$$c = \frac{\text{mg}_{(S+R_{\text{acid}})}}{\text{mg}_{(S+R_{\text{ester}})}} \times 100, \tag{2}$$

$$\text{ee}_{\text{p}} = \frac{S-R}{S+R} \times 100. \tag{3}$$

Here ee_{p} is the enantiomeric excess of the product and S and R represent the mass of (*S*)- and (*R*)-naproxen acids produced.

The enantioselectivity was calculated from the general equation [10]

$$E = \frac{\ln[1 - c(1 + \text{ee}_{\text{p}})]}{\ln[1 - c(1 - \text{ee}_{\text{p}})]}. \tag{4}$$

For low conversion values, this can be simplified to

$$E = \frac{S}{R}. \tag{5}$$

The standard deviation of data and related error were evaluated over three to five experiments.

3. Results and discussion

This section presents the experimental results obtained with the free and immobilized lipase used for the kinetic resolution of the three synthesized esters (methyl, butyl, and octyl naproxen esters). First, the native catalytic activity of lipase is characterized with its natural substrate to verify the native enzyme efficiency. Then the catalytic properties of lipase are studied using racemic esters with different –OR length groups synthesized in our laboratories. The different substrates were used with both the free and immobilized enzymes.

3.1. Catalytic properties of lipase with olive oil

Experiments with olive oil were carried out to evaluate its native catalytic performance. The experiments were carried out at 30 (±1) °C and pH 7.00. The reaction mixture was composed of 21 ml of phosphate buffer containing 1.02 mg of proteins (0.048 mg_{protein}/ml, with the value taking into account the ratio of protein with respect to the raw powder) and 1 ml of olive oil (acidity <1%).

The concentration of acid produced as a function of time is illustrated in Fig. 2 (with the error calculated over a series of five experiments). The enzyme had a reaction rate value of 3.2×10^{-2} ($\pm 8.6 \times 10^{-3}$) mmol/ml min and a specific activity of 2.1 (±0.55) $\text{mmol/min mg}_{\text{protein}}$.

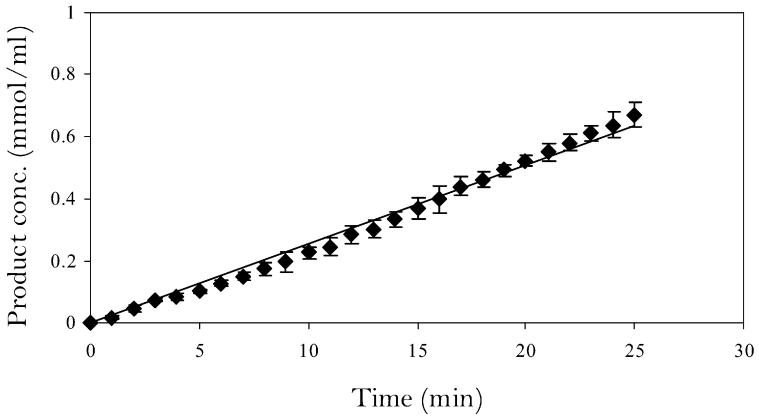


Fig. 2. Native catalytic performance of free lipase in the stirred tank reactor with triglycerides as substrate.

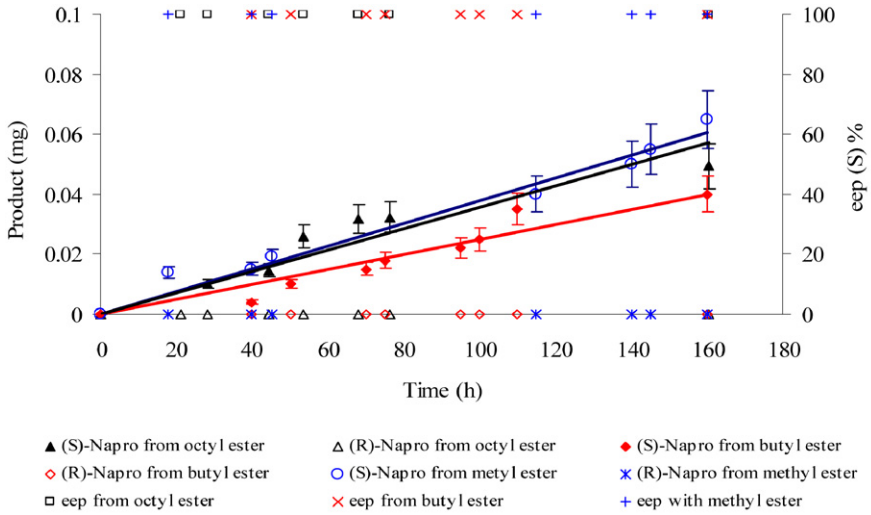


Fig. 3. Behavior of (*S*)- and (*R*)-naproxen acid and enantiomeric excess of free lipase in the stirred tank reactor with methyl, butyl, and octyl esters as substrates.

3.2. Catalytic properties of lipase with synthesized naproxen esters

Because the racemic ester with different –OR groups was not commercially available, the esters of interest were first synthesized and then used in kinetic resolution by means of free and immobilized lipase.

3.2.1. Catalytic properties of free lipase

The reaction mixture comprised 26 ml of 50 mM phosphate buffer (pH 7.00) containing 1.02 mg of proteins (0.039 $mg_{protein}/ml$) and 20 ml of 5 mM naproxen ester solution in isooctane. The reactions were carried out at 30 (±1) °C with stirring at 500 rpm. An overall error of 15% was associated with the experimental results calculated from three different experiments.

The enantioselective hydrolysis of naproxen methyl, butyl, and octyl esters by free lipase is shown in Fig. 3, which illustrates the production of (*S*)- and (*R*)-naproxen acid and the enantiomeric excess of the (*S*)-isomer as a function of time. The data show no production of (*R*)-naproxen acid during the observation period, indicating 100% enantiomeric excess of the (*S*)-acid and maximal enantioselectivity of the free lipase for the three naproxen esters was. For each ester, the amount of (*S*)-naproxen acid produced, the enzyme specific activity, and the reaction rate are summarized in Table 2a. The results show that the enzyme was more active with the methyl ester than for the other two esters. This implies that the interaction between the enzyme active site and the (*S*)-butyl and (*S*)-octyl esters were more restrictive (or specific) compared with the (*S*)-methyl ester—or, put another way, the interactions between the active site and the (*R*)-esters of butyl and octyl naproxen were more prohibitive. This was also confirmed by the fact that the lipase subjected to high shear stress, before the kinetic resolution reaction, exhibited decreased selectivity toward the (*S*)-methyl ester but unchanged activity toward the butyl and octyl esters.

3.2.2. Catalytic properties of immobilized lipase

The catalytic performance of the immobilized lipase was evaluated by monitoring the naproxen acid in the aqueous phase recycled along the lumen circuit of the two-separate-phase membrane reactor. The enzyme was entrapped within the asymmetric structure of the polymeric membrane, which separated the organic phase containing the substrate from the aqueous phase extracting the product.

Table 2
Catalytic performance and selectivity of lipase towards the enantioselective hydrolysis of racemic naproxen methyl, butyl, and octyl esters

(a) Lipase free in a stirred tank reactor

Substrate	Protein (mg)	c (%)	ee_p (%)	(S)-naproxen acid (mg)	Catalytic activity (mmol/min)	Reaction rate (μM/h)
(R,S)-naproxen methyl ester	1.02	3.4×10^{-1}	100	8.1×10^{-2} ($\pm 1.23 \times 10^{-2}$)	3.5×10^{-8} ($\pm 5.0 \times 10^{-9}$)	8.4×10^{-2} ($\pm 1.2 \times 10^{-2}$)
(R,S)-naproxen butyl ester	1.02	1.7×10^{-1}	100	4.2×10^{-2} ($\pm 0.63 \times 10^{-3}$)	1.8×10^{-8} ($\pm 2.0 \times 10^{-9}$)	4.3×10^{-2} ($\pm 6.4 \times 10^{-3}$)
(R,S)-naproxen octyl ester	1.02	1.1×10^{-1}	100	4.9×10^{-2} ($\pm 7.3 \times 10^{-3}$)	2.1×10^{-8} ($\pm 3.0 \times 10^{-9}$)	5.8×10^{-2} ($\pm 8.7 \times 10^{-3}$)
Triglycerides	1.02	–	–	–	2.1 ($\pm 1.0 \times 10^{-1}$)	1.92×10^{6} ($\pm 2.8 \times 10^{5}$)

(b) Lipase immobilized in a two-separate phase membrane reactor

Substrate	Immobilized protein (mg)	c (%)	ee_p (%)	(S)-naproxen acid (mg)	Catalytic activity (mmol/min)	Observed reaction rate (μM/h)
(R,S)-naproxen methyl ester	8.1	3.0	74	7.8 (± 1.17)	3.5×10^{-6} ($\pm 5.2 \times 10^{-7}$)	8.7×10^{-1} ($\pm 1.3 \times 10^{-1}$)
(R,S)-naproxen butyl ester	10.0	4.8	96	8.9 (± 1.33)	4.07×10^{-6} ($\pm 6.1 \times 10^{-7}$)	9.7×10^{-1} ($\pm 1.4 \times 10^{-1}$)
(R,S)-naproxen octyl ester	9.6	7.4×10^{-1}	100	3.3 (± 0.49)	1.48×10^{-6} ($\pm 2.2 \times 10^{-7}$)	4.3×10^{-1} ($\pm 6.4 \times 10^{-2}$)

Note. c = conversion; ee_p = enantiomeric excess of the (S)-naproxen acid produced.

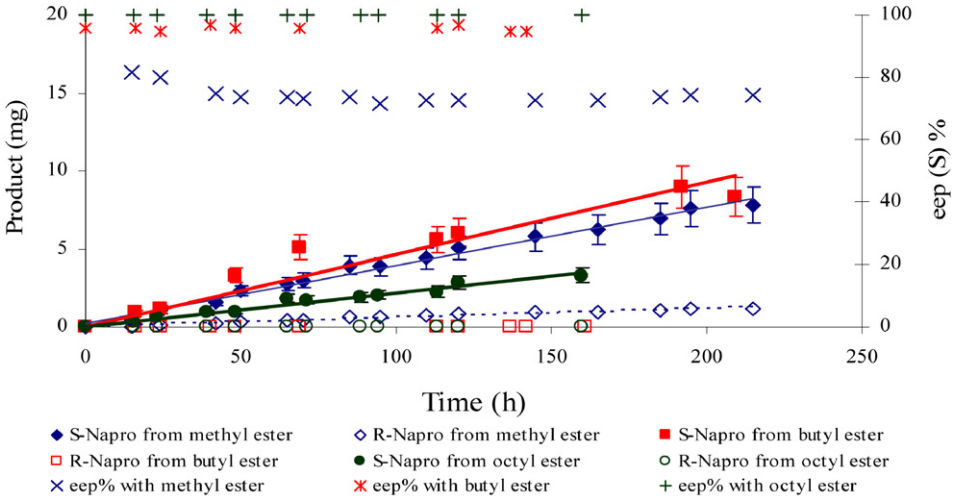


Fig. 4. Behavior of (S)- and (R)-naproxen acid and enantiomeric excess of immobilized lipase in the two-separate-phase membrane reactor during the hydrolysis of naproxen esters.

The organic phase was 200 ml of isooctane containing 5 mM naproxen ester and the aqueous phase was 250 ml of 50 mM phosphate buffer pH 7.00.

Fig. 4 illustrates the results obtained with the naproxen methyl, butyl, and octyl esters. As can be seen, when naproxen methyl ester was used as the substrate, the enantiomeric excess ($ee_p = 74\%$) was lower than that obtained from the reaction catalyzed by the free lipase (100%); see Fig. 3. Using the butyl ester, the enantiomeric excess ($ee_p = 96\%$) was still lower compared with that (100%) obtained by the free enzyme, but higher compared with that of naproxen methyl ester hydrolysis catalyzed by the immobilized lipase. Using the octyl ester, the enantiomeric excess was 100%, as was the case for the reaction with free lipase.

The results clearly demonstrate that the enantioselectivity of the immobilized lipase improved with the –OR group length. This meant that the immobilized enzyme underwent conformational rearrangements that opened the hydrophobic tunnel in the active site, making the catalytic triad Ser 209, His 449, Glu 341 more accessible and affecting the enantioselectivity in esters with low –OR length, but having a negative influence on enantioselectivity with the longer –OR chain esters. These conformational rearrangements had a positive effect on the catalytic activity; in fact, the observed catalytic activity normalized

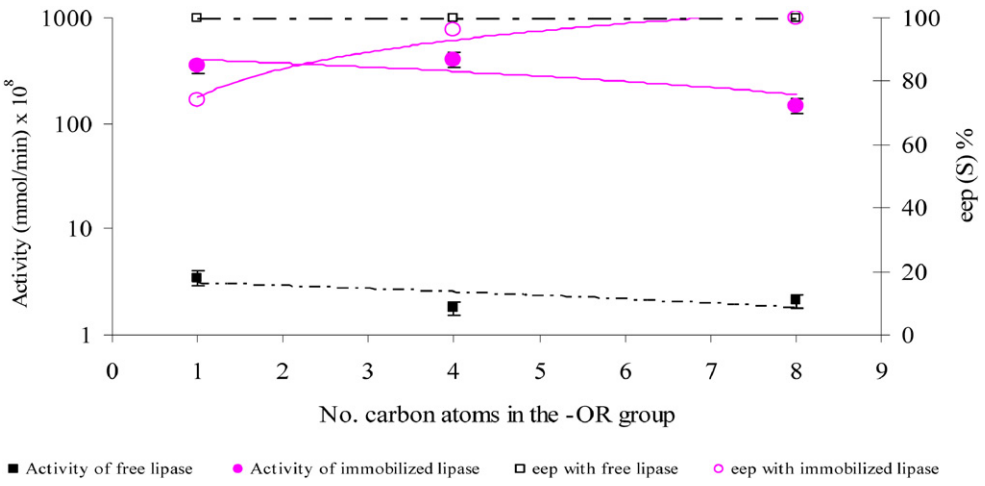


Fig. 5. Behavior of enantiomeric excess and catalytic activity as a function of number of carbon atoms in the –OR ester group for free and immobilized lipase.

by the mass of protein ($mmol_{product}/min\,mg_{protein}$) was higher for the immobilized enzyme than for the free one (see Table 2).

The increased reaction rate of immobilized lipase compared with free lipase may be due not only to the higher protein content in the membrane reactor, but also to the higher amount of lipase present in the protein loaded into the membrane (due to a partial purification during the immobilization process) and to the conformational rearrangements experienced by the immobilized lipase. The partial purification of immobilized lipase was deduced from the observation that during immobilization, some protein passed through the 50-kDa cutoff membrane and showed no hydrolytic activity with the olive oil. Electrophoresis analyses confirmed that the protein was of smaller molecular size than the 65-kDa of the lipase. The higher conversion obtained with the different esters in the enzyme-loaded membrane reactor than in the stirred tank reactor was due to the fact that the membrane reactor allowed removal of the product from the reaction site as it was formed, thus shifting equilibrium towards the product formation.

Fig. 5 illustrates the activity and enantiomeric excess of free and immobilized lipase as functions of carbon atoms in the –OR group. For both enzyme conditions, the activity decreased, increasing the number of carbon atoms. For the free lipase, the ee_p was constant; for the immobilized lipase, the ee_p increased with the number of carbon atoms.

4. Conclusion

This paper investigated the catalytic performance of free and immobilized lipase with three esters of naproxen with different –OR group lengths: methyl, butyl, and octyl esters. The results demonstrate that the free lipase was highly selective with all three esters and was most active with the shorter –OR group ester—the methyl ester. The lipase immobilized in the asymmetric membranes and used in the two-separate-phase membrane reactor improved the enantioselectivity with the –OR group length. The immobilized lipase showed higher observed activity per mass of protein than the free enzyme. The inverse relationship between enantioselectivity and activity as a function of number of carbon atoms in the –OR group for the immobilized enzyme implies that the selection of a suitable substrate for a productive system depends on the balance between the purity and productivity of the optically active enantiomer of interest.

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