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Short Communication

Immobilization of *Candida rugosa* lipase onto magnetic beads for kinetic resolution of (*R*,*S*)-ibuprofen

Michał Piotr Marszałł *, Tomasz Siódmiak

Department of Medicinal Chemistry, Collegium Medicum in Bydgoszcz, ul. Jurasza 2, 85-094 Bydgoszcz, Poland

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ABSTRACT

Two commercially available lipases from *Candida rugosa* (CRL from Sigma-Aldrich Co. and OF from Meito Sangyo Co.) were immobilized onto glutaraldehyde-activated and EDC/sulfo-NHS-activated amine-terminated magnetic beads (MB). In this study a procedure for immobilization of lipase OF using EDC and sulfo-NHS onto the surface of magnetic particles was developed. The resulting "OF lipase enzymatic system" yielded good results of enantioselectivity ($E = 19$, $ee_p = 83\%$) and conversion ($c = 42\%$) of the kinetic resolution of (*R*,*S*)-ibuprofen. Additionally, this procedure provides easy recovery and effective reuse of lipase OF, maintaining the enantioselectivity of the reaction on the same high level after five cycles. It was also demonstrated that the cross-linking reaction of lipases (CRL and OF) via glutaraldehyde onto magnetic support did not result in acceptable levels of conversion and enantioselectivity of the esterification reaction. Based on the results it should be noted that the immobilization technique we studied using EDC and sulfo-NHS onto MB could be potentially important for industrial application of kinetic resolution of non-steroidal anti-inflammatory drugs.

1. Introduction

Lipases from *Candida rugosa* (EC 3.1.1.3) are enzymes demonstrating various biological activities. The biocatalytic application of these enzymes has been reported in the literature as a potential approach in organic reactions, such as asymmetric esterification, asymmetric hydrolysis and asymmetric transesterification [1,2]. Numerous studies using lipases concern the kinetic resolution of 2-arylpropionic acids (the profens). These reactions are important from the pharmacological point of view because enantiomers of these drugs demonstrate different therapeutic activities, e.g. the (*S*)-enantiomer of ibuprofen is 160 times more active than its (*R*)-enantiomer in exerting in vitro inhibition of prostaglandin synthesis. Hence the application of the pure (*S*)-enantiomer instead of racemic ibuprofen allows for the reduction of the amount of total drug to achieve the expected therapeutic effect [3–5]. Additionally, kinetic resolution of profens by esterification yields an effective way to prepare the prodrugs. It is of special importance for drug action and pharmacokinetics because the major non-steroidal anti-inflammatory drugs (NSAIDs) can cause gastrointestinal side effects due to the direct contact of the acidic moiety of profens with the gastrointestinal mucosa [6,7].

Because of their low stability the application of lipases in the industry is limited. Many lipase immobilization techniques have therefore been employed [8–10]. The most important factors that should be taken into account in the selection of the immobilization strategy include: good catalytic activity, stability and reusability of the enzymes. Numerous reports on lipase immobilization in different supports have been published so far [11–14]. Most recently, the use of magnetic supports for the immobilization of different enzymes for ligand and protein "fishing" and their isolation or purification have been proposed [15]. The main advantage of micro- and nanomagnetic particles as a support for lipase immobilization is that the particles can be easily recovered from the reaction medium. Consequently, the use of magnetic particle supports can reduce the reaction costs, which might be of special importance for the chemical and pharmaceutical industry. In this context, various micro- and nanomagnetic particles have been evaluated in many studies [16–18]. Most of these studies have, however, been aimed to optimize the immobilization process mainly by glutaraldehyde cross-linking reaction and to characterize the size, structure, magnetic activity, amount and activity of the immobilized enzyme using the Bradford method and olive oil hydrolysis [19,20].

In the present study, the catalytic activity of two different *C. rugosa* lipases immobilized onto the amine-terminated magnetic particle support has been studied. The tested "enzyme magnetic particles" were assessed as potential enzyme systems for the esterification of racemic ibuprofen. Two cross-linking reactions via amine-domain (glutaraldehyde) and carboxyl-domain (carbodiimide) and their influence on the kinetic resolution of ibuprofen have been compared. Furthermore, the enantioselectivity of the esterification reaction after adding a salt hydrate pair and molecular sieves as well as the

* Corresponding author at: Collegium Medicum in Bydgoszcz, ul. Jurasza 2 85-094 Bydgoszcz, Poland. Fax: +48 52 585 3804.
E-mail address: mmars@cm.umk.pl (M.P. Marszałł).

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stability and catalytic activity of reused magnetically separated lipases has also been studied.

2. Experimental

2.1. Enzymes

Lipase CRL type VII from *C. rugosa* (activity ≥700 units/mg solid) was obtained from Sigma-Aldrich Co. (Germany). Lipase OF from *C. rugosa* (activity 380,000 units/g solid) was a gift from Meito Sangyo Co., LTD. (Japan).

2.2. Chemicals

Racemic (*R*,S)-ibuprofen, pure *S*(+)-enantiomer, *N*-(3-dimethylaminpropyl)-*N*′-ethylcarbodiimide (EDC), *N*-hydroxysulfosuccinimide sodium salt (sulfo-NHS), glutaraldehyde, and phosphate buffered saline were purchased from Sigma-Aldrich Co. (Stainhaim, Germany). 2-propanol, cyclohexane, *n*-hexane, acetic acid, pyridine, phosphoric acid, sodium sulfate anhydrous, sodium sulfate decahydrate, and molecular sieves 4 Å were purchased from POCH S.A. (Gliwice, Poland). The (*R*)- and (*S*)-esters of ibuprofen were obtained by the products of standard esterification reaction of (*R*,*S*)-ibuprofen and (*S*)-ibuprofen with 1-propanol using sulfuric acid (H_2SO_4) as catalyst [21].

Amine terminated magnetic beads (MB) (50 mg/mL, 1 μm) were purchased from Bioclone Inc. (San Diego, CA, USA). All supernatants were separated from magnetic beads (MB) using a magnetic separator Dynal MPC-S (Invitrogen Corporation, Carlsbad, CA, USA). Water used in the study was prepared using a Milli-QWater Purification System (Millipore, Bedford, MA, USA). All incubations were performed at adjusted temperatures and number of rotations in Thermomixer comfort (Eppendorf Co, Germany).

2.3. Instrumentation

The Shimadzu HPLC system (Japan) equipped with two solvent pumps model LC-20AD, UV–VIS detector model SPD-20A, degasser model DGU-20A_5, an autosampler model SIL-20AC_{HT} and a column oven model CTO-10AS_{VP}. Chiral Lux Cellulose-1 (4.6 mm × 250 mm × 5 μm) column with tris(3,5-dimethylphenylcarbamate) stationary phase, Chiral Lux Cellulose-2 (4.6 mm × 250 mm × 5 μm) column with tris(3-chloro-4-methylphenylcarbamate) stationary phase, Chiral Lux Cellulose-3 (4.6 mm × 250 mm × 5 μm) column with tris(4-methylbenzoate) stationary phase and Guard Cartridge System model KJO-4282 were purchased from Phenomenex Co.

2.4. Determination of optical purity and enantioselectivity

The enantiomeric excess of the substrate (ee_s) and the product (ee_p) as well as the conversion (c) and enantioselectivity (E) were calculated as below [22,23]:enantioselectivity (E):

$$E = \frac{In[(1-c)(1-ee_s)]}{In[(1-c)(1+ee_s)]} \quad (1)$$

the ee_s and ee_p values:

$$ee_s = \frac{R-S}{R+S} \quad (2)$$

$$ee_p = \frac{R-S}{R+S} \quad (3)$$

For R > S where S and R represent the chromatographic peak areas of the *S*- and *R*-enantiomers, respectively. The quantities of ibuprofen and ibuprofen esters were expressed by the value of the chromatographic peak areas.

The conversion (c):

$$c = \frac{ee_s}{ee_s + ee_p} \quad (4)$$

Additionally, in the presented results the ee_s and ee_p values are expressed in a percentage using the following equations:

$$ee_s = \frac{R-S}{R+S} \times 100 \quad (5)$$

$$ee_p = \frac{R-S}{R+S} \times 100 \quad (6)$$

The concentration (c) is also expressed in a percentage, using (5) and (6) for the calculation of conversion equations.

2.5. Chromatographic conditions

The effect of different compositions of the mobile phase consisted of three compounds: *n*-hexane, 2-propanol and acetic acid on the separation selectivity of (*R*)- and (*S*)-ibuprofen and their esters was investigated. Finally, the most appropriate chromatographic conditions were optimized with *n*-hexane/2-propanol/acetic acid (99.6/0.4/0.15 v/v/v) mobile phase at a flow rate of 1 mL/min. Three types of chiral chromatographic columns were tested, including Lux Cellulose-1, Lux Cellulose-2 and Lux Cellulose-3. With respect to the peak shape and the chiral resolution alike, the Lux Cellulose-1 (4.6 mm × 250 mm × 5 μm) HPLC column was chosen as an optimal one for the separation of (*R*)- and (*S*)-ibuprofen and their esters. The chromatographic process was operated at 25 °C. The detection UV wavelength was 254 nm.

2.6. Preparation of lipase-coated magnetic beads

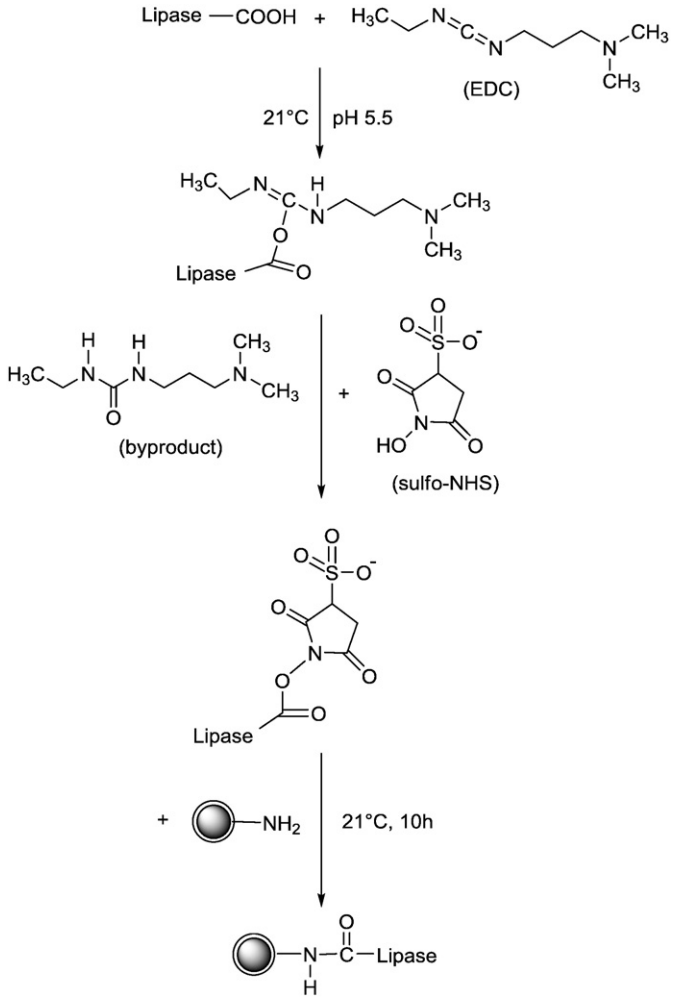
2.6.1. Covalent coupling of lipase using EDC and sulfo-NHS

The immobilization of lipase onto amine-terminated magnetic beads (MB) was performed by the formation of an amide bond between the carboxyl group of lipase and the primary amino group of the MB (Scheme 1). The preparation procedures were similar to those of previously described with the immobilization of melanin onto MB [24]. The 0.25 mL (12.5 mg) suspension of MB was placed into each of the four 1.5 mL centrifuge tubes and rinsed with 10 mM phosphate buffer (pH 5.5). Next, the four solutions of 10 mg lipase in 0.5 mL of 10 mM phosphate buffer (pH 5.5) (two solutions with OF lipase and two with CRL lipase) were prepared. 200 μL of a 10 mg/mL solution of EDC and sulfo-NHS was added to each of them and shaken for 5 min with gentle rotation and then transferred into separate centrifuge tubes along with the previously rinsed beads. Next, the resulting mixtures were shaken at 600 rpm in a thermomixer for 10 h at 21 °C. After incubation of the lipase-coated beads they were rinsed three times with 0.5 mL of water. Finally, the four mixtures were divided into two sets (four tubes). Each set was composed of one tube with OF lipase and the second tube with CRL lipase immobilized magnetic beads. The first set was air dried overnight. The second set was rinsed three times with 1 mL of 2-propanol, then with 1-propanol and finally with cyclohexane. Next, both sets were used in the esterification reaction.

2.6.2. Covalent coupling of lipase using glutaraldehyde

The immobilization of lipase onto amine-terminated magnetic beads (MB) was performed by the formation of a bond between the primary amino group of lipase and the aldehyde groups of glutaraldehyde (Scheme 2).

The immobilization of lipase using glutaraldehyde and 10 mM pyridine solution was performed on the surface of amine-terminated



Scheme 1. Immobilization of lipase using EDC and sulfo-NHS onto the surface of magnetic beads (MB).

magnetic beads using the protocol provided by Bioclone Inc with slight modifications. 0.25 mL (12.5 mg) of suspension of MB was placed into each of the four 1.5 mL centrifuge tubes and rinsed with 10 mM pyridine solution (pH 6.0). Next, the 0.5 mL of 5% glutaraldehyde solution was added to all tubes and shaken at 600 rpm in a thermomixer for 0.5 h at 21 °C. After that time MB were rinsed again with 10 mM pyridine solution (pH 6.0). Next, the four solutions of 10 mg lipase in 0.5 mL of 10 mM pyridine solution (pH 6.0) were prepared (two solutions with OF lipase and two with CRL lipase). The resulting four solutions were placed in separate centrifuge tubes and mixed with the rinsed beads. The mixtures were then shaken at 600 rpm in a thermomixer for 6 h at 21 °C. After incubation the lipase-coated beads were rinsed three times with 0.5 mL of pyridine solution. The four mixtures were divided into two sets, as in the previous immobilization procedure. Each set was composed of one tube with OF lipase and a second tube with CRL lipase immobilized onto the surface of magnetic beads. The first set after being rinsed with 0.5 mL of water was air dried overnight. The second set was rinsed three times with 0.5 mL of water, then with 1 mL of 2-propanol, 1-propanol and finally with cyclohexane. Next, the prepared magnetic beads were used in the esterification reaction.

2.7. Lipase-catalyzed esterification of (R,S)-ibuprofen

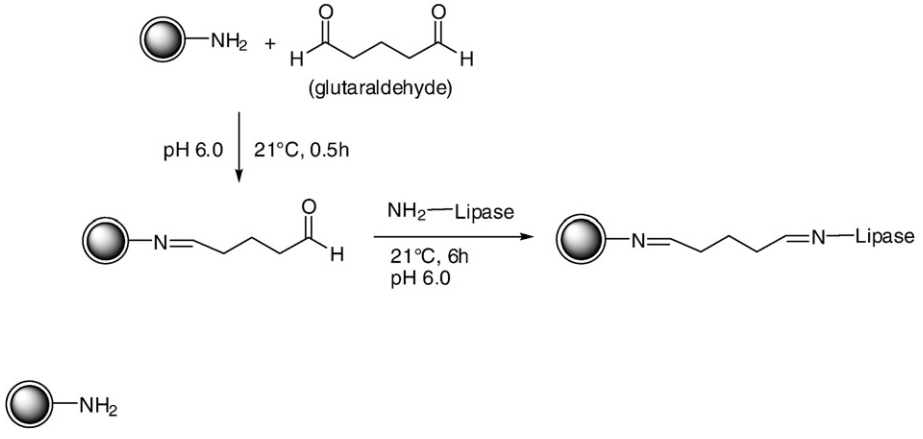
The reaction mixture was composed of cyclohexane (700 μL), racemic ibuprofen (8.25 mg, 0.04 mM) and 1-propanol (9 μL, 0.12 mM) as an acyl acceptor. The reaction was started by adding this solution to the magnetic beads with immobilized lipase in a 1.5 mL tube. The suspension was incubated at 37 °C, shaken (600 rpm) for 144 h in a thermomixer. The samples (50 μL) were withdrawn after 60 h and 144 h. The collected supernatant was removed by evaporation at room temperature and the residue was dissolved in 0.7 mL mobile phase and injected (25 μL) into HPLC. The esterification reaction of racemic ibuprofen with 1-propanol is shown in Scheme 3.

3. Results and discussion

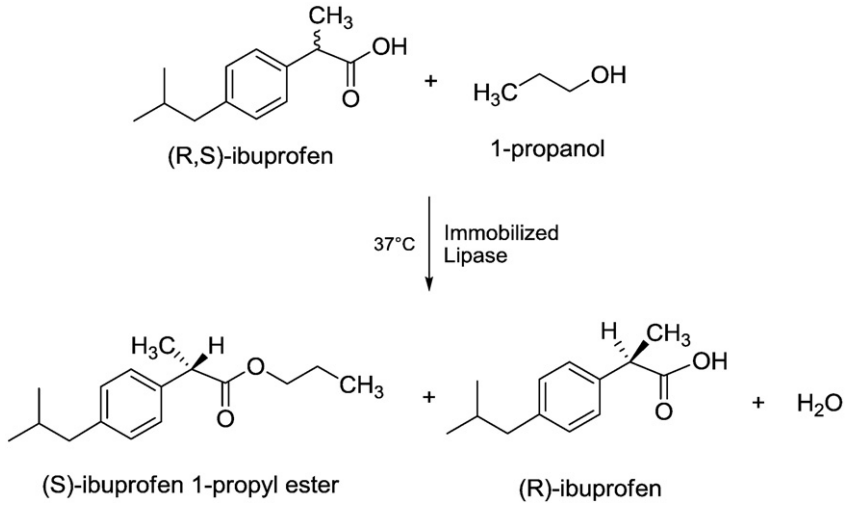
3.1. Application of immobilized lipases in kinetic resolution of (R,S)-ibuprofen

Immobilization of commercially available lipases from *C. rugosa* (OF and CRL) was performed by two different procedures via glutaraldehyde and EDC/sulfo-NHS cross-linking reaction. Due to the different treatment of the resulting magnetic beads in the last step of the immobilization process (air drying or rinsing with 2-propanol, 1-propanol and finally cyclohexane) different effects on the esterification of (*R,S*)-ibuprofen were observed. As shown in Table 1, the immobilization procedure using EDC and sulfo-NHS with air drying yields acceptable results for enantioselectivity (E) and conversion degree (C) for OF lipase. The markedly weak values of the conversion of the kinetic resolution of (*R,S*)-ibuprofen were achieved for CRL lipase immobilized in the same manner.

The application of procedure EDC/sulfo-NHS and air drying allowed to obtain three times higher value of enantioselectivity (E) for CRL lipase and two times higher E-value for OF lipase than using glutaraldehyde. After 144 h of the reaction, for all



Scheme 2. Immobilization of lipase onto the surface of magnetic beads (MB) using glutaraldehyde cross-linking reaction.



Scheme 3. The enantioselective esterification of racemic ibuprofen with 1-propanol with the use of immobilized lipase onto the surface of magnetic beads as biocatalyst.

performed procedures the conversion was two times higher in comparison with values after 60 h (Table 1a and b).

It should be emphasized that OF and CRL lipases immobilized with the use of the glutaraldehyde cross-linking reaction demonstrate low enantioselectivity and conversion of esterification, both for air dried samples and samples rinsed with 2-propanol. Application of 2-propanol in EDC/sulfo-NHS immobilization procedure also gives low E-value and conversion for studied lipases. Supposedly, the low values of enantioselectivity obtained in case of 2-propanol in the last step of immobilization are the result of dehydration of enzyme and the resulting significant loss of its catalytic activity. Hence, the further aim of the study was to improve the enantioselectivity and conversion of esterification of racemic ibuprofen catalyzed by OF lipase that was immobilized onto magnetic beads support with the use of EDC/sulfo-NHS and air drying procedure.

Table 1
The influence of immobilization procedure on the conversion and enantioselectivity of esterification reaction of (*R,S*)-ibuprofen after 60 (a) and 144 h (b).

Time [h]	Lipase	Immobilization procedure	ee_p (%)	ee_s (%)	C (%)	E
a)						
60	OF	Glutaraldehyde, air drying	40.2	1.9	4.6	2.4
60	CRL	Glutaraldehyde, air drying	30.6	0.2	0.6	1.9
60	OF	EDC, sulfo-NHS, air drying	57.2	13.2	18.8	4.2
60	CRL	EDC, sulfo-NHS, air drying	74.0	0.7	0.9	6.5
60	OF	Glutaraldehyde, 2-propanol	53.0	2.1	3.8	3.3
60	CRL	Glutaraldehyde, 2-propanol	42.4	0.2	0.6	2.4
60	OF	EDC, sulfo-NHS, 2-propanol	45.1	0.6	1.4	2.7
60	CRL	EDC, sulfo-NHS, 2-propanol	38.9	0.2	0.5	2.3
b)						
144	OF	Glutaraldehyde, air drying	41.1	3.7	8.4	2.5
144	CRL	Glutaraldehyde, air drying	40.2	0.2	1.2	2.3
144	OF	EDC, sulfo-NHS, air drying	47.5	36.0	43.1	4.0
144	CRL	EDC, sulfo-NHS, air drying	71.2	1.8	2.5	6.0
144	OF	Glutaraldehyde, 2-propanol	51.2	4.3	7.7	3.2
144	CRL	Glutaraldehyde, 2-propanol	44.6	0.6	1.3	2.6
144	OF	EDC, sulfo-NHS, 2-propanol	44.6	1.5	3.3	2.6
144	CRL	EDC, sulfo-NHS, 2-propanol	40.5	0.5	1.2	2.3

Reaction conditions: (*R,S*)-ibuprofen (0.04 mM), 1-propanol (0.12 mM), immobilized lipase CRL or OF (12.5 mg of magnetic beads), cyclohexane (700 μL), temp. 37 °C; C — conversion, ee_s — enantiomeric excess of the substrate, ee_p — enantiomeric excess of the product, E — enantiomeric ratio.

3.2. Effect of salt hydrate and molecular sieves on the enantioselectivity of lipase OF immobilized onto magnetic beads support

The effect of water activity on the esterification reaction was controlled through direct addition of a salt hydrate pair $Na_2SO_4/Na_2SO_4 \cdot 10\text{-}H_2O$ (35 mg in total, with molar ratio of 1:1) into 0.7 mL of cyclohexane containing (*R,S*)-ibuprofen (8.25 mg, 0.04 M) and 1-propanol (9 μL, 0.12 mM). The resulting mixture was shaken for 0.5 h at 37 °C and 600 rpm. After that time, the immobilized lipase OF (12.5 mg of magnetic beads) was added and the mixture was incubated at 37 °C and 600 rpm in thermomixer. Additionally, the molecular sieves 4 Å after 3 h were added in order to improve the adsorption of water from the reaction medium (as a byproduct).

Water plays a critical role in the structure and function of enzymes because of its influence on the active conformation of enzymes. It is therefore necessary to maintain the correct water activity in biocatalytic reactions. Salt hydrates provide crystallization water to the components and simultaneously adsorb water generated during the esterification reaction. Additionally, the presence of molecular sieves in the reaction medium improves the adsorption of water. The application of salt hydrates and molecular sieves allowed us to achieve a higher enantioselectivity ($E = 19$, $ee_p = 83\%$, $c = 42\%$ for immobilized lipase OF after 144 h) compared to the results obtained in the absence of these additives ($E = 4.0$, $ee_p = 47.5\%$, $c = 43.1\%$ for immobilized lipase OF, after 144 h).

3.3. Reuse of immobilized lipase in the kinetic resolution of (R,S)-ibuprofen

The reusability of the immobilized lipase is very important from the commercial point of view. In order to test the efficiency of immobilized lipase OF (12.5 mg) the same beads were reused after the specified washing procedure. After the first cycle of the esterification reaction the used immobilized lipase was recovered from the reaction medium by magnetic separation and washed three times with cyclohexane. Next, the lipase-coated beads were air dried overnight to remove the organic solvent and then the beads were placed into a fresh medium containing a mixture of 1-propanol (9 μL, 0.12 mM), (*R,S*)-ibuprofen (8.25 mg, 0.04 mM), salt hydrate pair $Na_2SO_4/Na_2SO_4 \cdot 10\text{-}H_2O$ (35 mg in total) and molecular sieves 4 Å in cyclohexane (700 μL). The reaction was repeated up to five cycles with the same magnetic beads to determine the enantioselectivity of the esterification reaction (Fig. 1). During the five cycles of the kinetic resolution

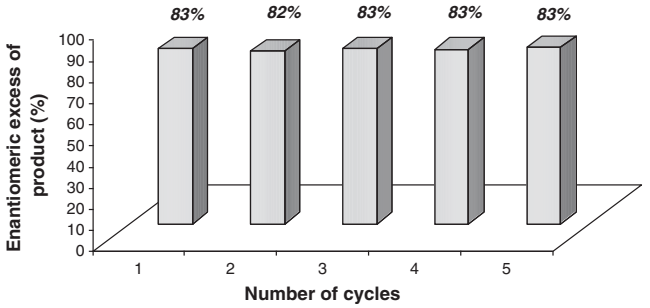


Fig. 1. Effect of repeated use of immobilized lipase OF onto surface of MB on the enantioselectivity of the esterification of (*R*,*S*)-ibuprofen. Reaction conditions: (*R*,*S*)-ibuprofen (0.04 mM), 1-propanol (0.12 mM), immobilized lipase OF (12.5 mg of magnetic beads), salt hydrate pair $Na_2SO_4/Na_2SO_4 \cdot 10H_2O$ (totally 35 mg, with molar ratio of 1:1), molecular sieves 4 Å, cyclohexane (700 μL), temp. 37 °C, after 144 h.

of racemic ibuprofen slight changes of the enantioselectivity of the reaction expressed as enantiomeric excess of product were observed. The results demonstrate that the EDC/sulfo-NHS cross-linking reaction is a very effective immobilization procedure that maintained the stability and catalytic activity of lipase after 5 cycles (30 days).

4. Conclusions

The study compared the catalytic activity of two commercially available *C. rugosa* lipases (CRL and OF) immobilized onto glutaraldehyde-activated and sulfo-NHS/EDC-activated amine-terminated magnetic beads. The immobilized lipases were used for the kinetic resolution of (*R*,*S*)-ibuprofen by enantioselective esterification. In all the cases, (S)-enantiomer was preferred by the studied lipases.

In summary, the most effective conversion and the best enantioselectivity was achieved by using lipase OF immobilized onto the magnetic support with EDC/sulfo-NHS and air drying procedure. The presence of a salt hydrate pair and molecular sieves in the reaction medium allowed us to achieve a higher enantioselectivity and conversion degree compared to the results obtained in the absence of these additives. What is crucial is that, the immobilized lipase is recoverable magnetically and can be effectively reused in the enantioselective esterification of (*R*,*S*)-ibuprofen. The easy removal of the "magnetic enzyme" particles from the reaction media offers an important advantage from the economic point of view. The studied magnetic support might be of special importance for the industrial application of the kinetic resolution of nonsteroidal anti-inflammatory drugs.

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