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Esterification of (*RS*)-Ibuprofen by native and commercial lipases in a two-phase system containing ionic liquids

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Abstract—Four commercially available lipases and two native lipases from *Aspergillus niger* AC-54 and *Aspergillus terreus* AC-430 were used for the resolution of (*RS*)-Ibuprofen in systems containing the ionic liquids [BMIM][PF_6] and [BMIM][BF_4]. The lipases showed higher conversion in a two-phase system using [BMIM][PF_6] and isooctane compared to that in pure isooctane. Although the best enzyme was a commercially available lipase from *Candida rugosa* ($E = 8.5$), another native lipase, produced in our laboratory, from *A. niger* gave better enantioselectivity ($E = 4.6$) than the other lipases tested ($E = 1.9$–3.3). After thorough optimization of several reaction conditions (type and ratios of isooctane/ionic liquid, amount of enzyme, and reaction time), the *E*-value of *A. niger* lipase (15% w/v) could be duplicated ($E = 9.2$) in a solvent system composed of [BMIM][PF_6] and isooctane (1:1) after 96 h of reaction.

1. Introduction

Lipases are very used in several industrial applications because of their ability in catalyzing different reactions such as hydrolysis, esterification, and transesterification.[1] These enzymes are capable of recognizing the enantiomers of a racemate resulting in an enantiopure compound.[2] The use of lipases in an organic solvent makes synthetic reactions possible, that do not occur in the natural media of these enzymes. However, these solvents can have disadvantages, such as being volatile and toxic for the environment,[3] particularly when they are used on a large scale. An alternative for these organic solvents is the use of ionic liquids (ILs). These are salts that have one or both of the large ions, an organic cation and an inorganic anion, often fluid at room temperature. These solvents possess several interesting properties, such as ease of preparation, reuse, high thermal stability, and low vapor pressure and the possibility to manipulate at will its structure using different cations or anions.[4,5] The most commonly used ILs are 1-butyl-3-methyl imidazolium hexafluorophosphate ([BMIM][PF_6]) and tetrafluoroborate ([BMIM][BF_4]). Different enzymes, such as hydrolases have been shown to keep their activity and enantioselectivity in some ILs.[6,7] Moreover, in some cases they are higher than in some common organic liquids.[8,9]

Ibuprofen is marked as racemic drug, but its activity belongs to the (*S*)-(+)-enantiomer. It has been reported that (*S*)-(+)-Ibuprofen is 160-fold more active than its antipode in the synthesis of prostaglandin 'in vitro'.[10] Based upon this, many studies has been made attempting to resolve (*RS*)-Ibuprofen by different reactions, such as esterification,[11] transesterification,[12] and hydrolysis.[13] Lipases from *Candida rugosa* (*cylindracea*), *Candida antarctica* (Novozyme 435) and *Rhizomucor miehei* have been used to resolve the enantiomers of Ibuprofen,[14,15] naproxen,[16,17] and ketoprofen[18,19] in organic solvents. A few authors such as Hongwei et al.[20] have exploited lipase enantioselectivity in the resolution of (*RS*)-Ibuprofen in solvent systems containing ILs. Herein, we report the performance of native and commercial lipases in the enantioselective esterification of Ibuprofen with 1-propanol in systems containing the ILs [BMIM][PF_6] and [BMIM][BF_4], with the results compared to those in pure isooctane. Selected enzymes were optimized with respect to the type of IL, ratios between isooctane and [BMIM][PF_6], amount of enzymes, and reaction time.

2. Results and discussion

2.1. Enzyme screening

To study the influence of the ionic liquids (ILs) in the resolution of (*RS*)-Ibuprofen catalyzed by lipases, we

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designed an experiment wherein [BMIM][PF_6] and [BMIM][BF_4] were added as an additive (co-solvent) to the solvent isooctane. In the first stage, this experiment was carried through using equal parts of IL (250 μL) and isooctane (250 μL). This results in a system of two-phases, since isooctane is immiscible with the ILs employed. Enzymatic reactions in pure isooctane systems were also carried out in order to compare the efficiency of ILs for use as reaction media. Figure 1 shows the esterification activity expressed as a conversion degree (%) exhibited by both lipases after 48 h of reaction.

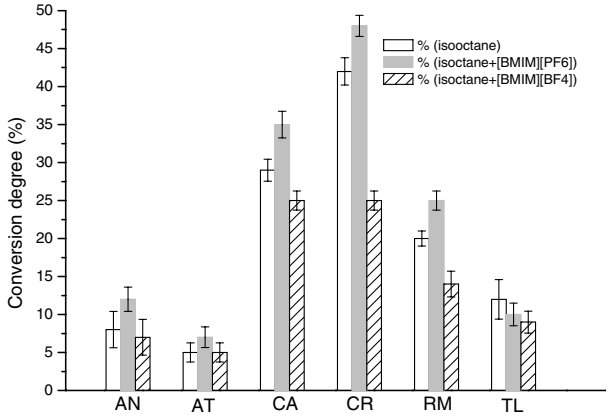

Figure 1. Conversion degree (%) of the resolution of (*RS*)-Ibuprofen obtained in the screening of several lipases in pure isooctane, isooctane + [BMIM][PF_6] (1:1) and isooctane + [BMIM][BF_4] (1:1). Lipase abbreviations: RM: *Rhizomucor miehei* (Lipozyme RM IM); TL: *Thermomyces lanuginose* (Lipozyme TL IM); CA: *Candida antarctica* type B (Novozyme 435), CR: *Candida rugosa*, AN: *Aspergillus niger* AC-54, and AT: *A. terreus* AC-430.

The most efficient lipases in the esterification of (*RS*)-Ibuprofen were from *C. rugosa* (CR), *C. antarctica* (CA), and *R. miehei* (RM). The highest conversion degree (48%, 35%, and 25%, respectively) was obtained when two-phase system containing [BMIM][PF_6] + isooctane (1:1) was used. The conversion degree increased 20–25% compared to using pure isooctane as solvent media. The activity of the native lipase from *Aspergillus niger* (AN) increased significantly (improved ~50% compared to that in isooctane) in a solvent system containing [BMIM][PF_6]. This was not observed when the mixture [BMIM][BF_4] + isooctane was used. In some cases such as RM and CR lipases, this IL was harmful to the enzyme activity (reduction ~40% compared to that in pure isooctane). The two liquids employed for the current study, [BMIM][PF_6] and [BMIM][BF_4], are distinctly different in their properties, such as hydrophobicity, polarity, anion nucleophilicity, hydrogen-bond basicity, and viscosity.[3] The [BMIM][BF_4] is highly hydrophilic in nature, whereas [BMIM][PF_6] is highly hydrophobic due to the different anions associated with the common organic cation. These properties are of primary concern in enzyme-catalyzed reactions, since they are capable of influencing the conformation of the lipases and consequently their reactivity.[3,21] The conversion degree in the resolution of (*RS*)-Ibuprofen reduced in the presence of [BMIM][BF_4] probably due to stripping of the essential water of the lipases.[6] However, this area is very new and much effort is needed in order to understand the effect of different ionic liquids in biotransformation reactions.

As shown in Table 1, the conversion degree (*c*), the enantiomeric excess (ee), and enantioselectivity (*E*-value) of the different lipases in the esterification of the (*RS*)-Ibuprofen in absence or presence of [BMIM][PF_6] were rather heterogeneous.

The best conversion degree (48%), enantiomeric excess of remaining acid (60%), and the *E*-value (8.5) were attained with the *C. rugosa* lipase in a two-phase system containing isooctane + [BMIM][PF_6] (1:1) compared with a system in pure isooctane. The conversion degree in the presence of an ionic liquid was not significantly improved (42% to 48%), but the *E*-value was doubled (4.1 to 8.5). The same behavior was observed using a lipase native of *A. niger*. The *E*-value was significantly increased (2.1 to 4.6) in the presence of [BMIM][PF_6]. As described in our previous works,[11] this lipase showed a stereopreference toward the (*R*)-(−)-enantiomer and a moderate *E*-value has been achieved, which is considerably higher than those results previously reported using this lipase.[18,22]

Table 1. Kinetic resolution of (*RS*)-ibuprofen catalyzed by native and commercial lipases in reaction media in pure isooctane or isooctane + [BMIM][PF_6] (1:1) after 48 h of reaction

Lipases	Solvent	*c* (%)	ee (%)	*E*	Stereo preference
Aspergillus niger AC-54	Isooctane	8	3	2.1[a]	*R*
	Isooctane + [BMIM][PF_6]	12	8	4.6[b]	*R*
Candida antarctica	Isooctane	29	18	3.1[a]	*R*
	Isooctane + [BMIM][PF_6]	35	24	3.3[a]	*R*
Candida rugosa	Isooctane	42	35	4.1[a]	*S*
	Isooctane + [BMIM][PF_6]	48	60	8.5[b]	*S*
Rhizomucor miehei	Isooctane	20	10	2.6[a]	*R*
	Isooctane + [BMIM][PF_6]	25	13	2.7[a]	*R*
Thermomyces lanuginosa	Isooctane	12	3	1.7[a]	*R*
	Isooctane + [BMIM][PF_6]	10	4	1.9[a]	*R*

c = Conversion is given as the percentage of initial racemic ibuprofen esterified after the reaction time, ee = enantiomeric excess of the (*S*)-Ibuprofen active, *E* = enantiomeric ratio.

Reaction conditions: (*RS*)-Ibuprofen (4 mM), 1-propanol (12 mM), lipase (5.0% w/v), pure solvent (250 μL), or mixture of organic solvent/ionic liquids (1:1), 35 °C, 300 rpm, 48 h.

The *E*-value means for each lipase with different superscript letters are significantly different $p \leqslant 0.05$.

R. miehei and *C. antarctica* lipases displayed a good conversion, but poor enantioselectivity, since they catalyzed the esterification of both the (*R*)- and (*S*)-enantiomers of Ibuprofen. These results indicated that commercial *C. rugosa* and native *A. niger* lipases exhibited the highest esterification activity and enantioselectivity in the presence of [BMIM][PF_6] and were therefore chosen as the model enzymes for continuity of this study.

2.2. Influence of ratios between isooctane and [BMIM][PF_6]

The amount of [BMIM][PF_6] was progressively increased from 0% to 100% v/v in the reaction mixture and the conversion and enantiomeric excess were monitored after 48 h (Table 2).

Table 2. Influence of ratios between isooctane/[BMIM][PF_6] (%) in the kinetic resolution of (*RS*)-Ibuprofen catalyzed by *Aspergillus niger* and *Candida rugosa* lipases

Isooctane/[BMIM][PF_6] (%)	*Aspergillus niger* lipase			*Candida rugosa* lipase		
	c (%)	ee (%)	*E*	*c* (%)	ee (%)	*E*
100/0	8	3	2.1[a]	42	35	4.1[a]
70/30	13	7	3.2[b,c]	45	56	9.1[b]
50/50	12	8	4.6[d]	48	60	8.5[b]
30/70	18	13	4.1[b,d]	38	36	5.3[a]
0/100	9	5	3.1[c]	38	34	4.7[a]

Reaction conditions: (*RS*)-Ibuprofen (4 mM), 1-propanol (12 mM), lipase (5.0% w/v), isooctane + [BMIM][PF_6] (500 μL), 35 °C, 48 h.
E-value means for each lipase with different superscript letters are significantly different $p \leqslant 0.05$.

The results indicate that the resolution of Ibuprofen occurs more easily in the biphasic system, with isooctane and [BMIM][PF_6] than in pure ionic liquid or pure isooctane. The best *E*-values were achieved by *C. rugosa* lipase at 30% [BMIM][PF_6] ($E = 9.1$) and 50% [BMIM][PF_6] ($E = 8.5$). For lipase from *A. niger*, a good result was found using 50% of the [BMIM][PF_6]. Similar results were found using 70% of the [BMIM][PF_6]. This indicates that the presence of both isooctane and [BMIM][PF_6] in the reaction mixtures, on the one hand causes the solubility of the drug to increase, while on the other hand the esterification causes the reaction to proceed with higher enantioselectivity compared to reactions in conventional solvents. This IL, despite being polar due its ionic nature, is hydrophobic, contrary to what is observed in the case of most of the common polar organic solvents.[6] This can justify the highest enantioselectivity compared with pure isooctane, since the enzyme needs a small amount of water to maintain its structure.

It has previously been reported that the enantioselectivity of lipases in different reactions can be increased by the use of ionic liquids,[23,24] while in other cases the enantioselectivity was similar in ionic liquid and in organic solvents.[25,26] Hongwei et al.[20] recently described a kinetic resolution of Ibuprofen catalyzed by *C. rugosa* lipase conducted in seven pure ionic liquids. Only in the case of [BMIM][PF_6] was the *E*-value ($E = 24.1$) almost twice that ($E = 13.0$) in isooctane.

Ionic liquids have been studied as pure solvents[27] and in a two-phase system with another solvent such as organic solvent[24] and with supercritical carbon dioxide.[28] Ganske and Bornscheuer,[29] reported that *C. antarctica* lipase (CALB-B Chirazyme L2) do not show activity in the synthesis of sugar esters in pure ionic liquids. However, it was possible using a biphasic mixture containing 60% ionic liquid ([BMIM][PF_6] or [BMIM][BF_4]) and 40% of *t*-butanol as solvent for the esterification reaction.

2.3. Influence of time reaction

Table 3 shows the conversion degree, the enantiomeric excess of remaining acid, and the *E*-values as a function of time reaction. The reactions were catalyzed by *A. niger* and *C. rugosa* lipases in a solvent system composed of 50% and 30% of [BMIM][PF_6], respectively.

For *A. niger* lipase, in the range of 24–96 h, the conversions were 6% and 15%, with enantiomeric excesses of 3% and 12%, resulting in *E*-values of 2.4 and 6.4. For lipase *C. rugosa*, after 24 h, more than 40% Ibuprofen propyl ester is synthesized in a solvent system composed of 70% of isooctane and 30% of [BMIM][PF_6], resulting in the *E*-value of 9.3 with no further increase after prolonged reaction times.

Table 3. Influence of reaction time in the kinetic resolution of (*RS*)-Ibuprofen catalyzed by *Aspergillus niger* and *Candida rugosa* lipases

Reaction time (h)	*Aspergillus niger* lipase			*Candida rugosa* lipase		
	c (%)	ee (%)	*E*	*c* (%)	ee (%)	*E*
12	6	0	ND	36	33	5.1[a]
24	6	3	2.4[a]	42	51	9.3[b]
48	12	8	4.6[b]	45	56	9.1[b]
72	14	10	4.9[b]	44	53	8.9[b]
96	15	12	6.4[c]	40	47	8.9[b]

ND: not detected.
Reaction conditions: (*RS*)-Ibuprofen (4 mM), 1-propanol (12 mM), lipase (5.0% w/v), isooctane + [BMIM][PF_6] (500 μL), 35 °C.
E-value means for each lipase with different superscript letters are significantly different $p \leqslant 0.05$.

2.4. Effect of enzyme loading

The influence of the amount of lipases on the resolution of (*RS*)-Ibuprofen in biphasic system is shown in Figures 2 and 3. The amounts used were 5%, 15%, 30%, and 50% (w/v) and the products were determined after 96 and 24 h in reactions catalyzed by *A. niger* and *C. rugosa* lipases, respectively.

Using 5% and 15% (w/v) of lipase from *A. niger* the conversion was 15% and 19%, with enantiomeric excesses of 12% and 17%, resulting in *E*-value of 6.4 and 9.2, respectively. Using 30% and 50% (w/v), better conversions (32% and 40%) and ee values (28% and 31%) were obtained. However, the *E*-values were 5.2 and 3.7 indicating the low selectivity of *A. niger* lipase under these experimental conditions. On the other hand, Ibuprofen can be obtained

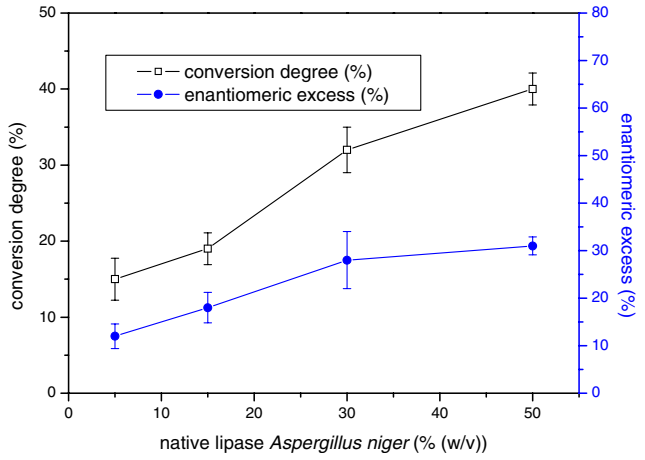

Figure 2. Effect of amount of *Aspergillus niger* lipase (% w/v) in the kinetic resolution of (*RS*)-Ibuprofen in solvent system composed of 50% of isooctane and 50% of [BMIM][PF_6] after 96 h.

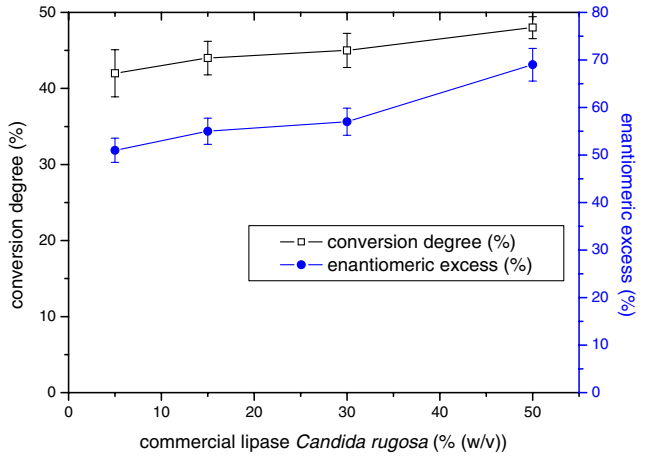

Figure 3. Effect of the amount of *Candida rugosa* lipase (% w/v) in the kinetic resolution of (*RS*)-Ibuprofen in solvent system composed of 70% of isooctane and 30% of [BMIM][PF_6] after 24 h.

with good ee (69%) and *E*-value (12) quenching the reaction near 50% conversion after 24 h reaction using 50% (w/v) of *C. rugosa* lipase (Fig. 3).

3. Conclusion

Although the *E*-values throughout this study were not high enough for industrial application, the significant increase in the enantioselectivity in the PF_6 anion containing ionic liquids is promising in a biocatalysts reaction especially when we consider the fact that ionic liquids are considered as green solvents and can be reused.

4. Experimental

4.1. Enzymes and chemical

Immobilized lipases from *R. miehei* (Lipozyme RM IM), *Thermomyces lanuginosa* (Lipozyme TL IM), and *C. antarctica* type B (Novozyme 435) were donated by Novozymes Latin America Ltda (Araucária, PR, Brazil). *C. rugosa* lipase was purchased from Sigma Chemicals (St. Louis, MO, USA) and native lipases from *A. niger* AC-54 (lipase activity 0.56 μmol min^{-1} mg^{-1} for the hydrolysis of olive oil at pH 7.0 and 40 °C) and *Aspergillus terreus* AC-430 (lipase activity 0.47 μmol min^{-1} mg^{-1} for the hydrolysis of olive oil at pH 6.0 and 40 °C) were obtained in our laboratory[30] and stored from the culture collection of the Laboratory of the University São Francisco (Brazil). Ionic liquids, 1-butyl-3-methyl imidazolium tetrafluoroborate [BMIM][BF_4], 1-butyl-3-methyl imidazolium hexafluorophosphate [BMIM][PF_6], isooctane, 1-propanol, and (*S*)-Ibuprofen were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). All other chemical reagents were obtained from Merck (Darmstadt, Germany) or Sigma–Aldrich Chemical Co. in the highest purity available.

4.2. General procedure for enzymatic reactions

In a typical experimental procedure, 4 mM (*RS*)-Ibuprofen and 12 mM 1-propanol were placed into screw-capped vials and dissolved in 250 μL of the isooctane. Five percent (w/v) enzyme, ten percent (w/v) activated molecular sieves (4 Å), and 250 μL of the ionic liquids [BMIM][PF_6] (1-butyl-3-methyl imidazolium hexafluorophosphate) or [BMIM][BF_4] (1-butyl-3-methyl imidazolium tetrafluoroborate) were added. The reaction mixtures were shaken in a New Brunswick with orbital magnetic stirring at 300 rpm at 35 °C. At regular time intervals, 500 μL of the isooctane was added and the biphasic mixture strongly shaken for 3 min to extract all substrates and products into the isooctane phase. Then, 50 μL aliquots of the resultant solution were withdrawn in the isooctane phase and the amount of ester (conversion degree) formed during the reaction and the enantiomeric excess of the (*S*)-enantiomer determined by GC and by HPLC, respectively, are as described below.

4.3. Chromatography analysis

Gas chromatography was performed using a CHROMPACK CP 9001 gas chromatography equipped with flame ionization detector (FID) and a CP-Sil 5 CB column (10 m × 0.25 mm × 0.12 μm). Injector temperature was 300 °C and the detector was 350 °C; an oven temperature was maintained at 150 °C. The carrier gas was hydrogen with a flow rate of 12 mL/min. An external standard method was employed to quantify the formed ester and the remaining acid. The enantiomers of the unreacted substrate were separated by HPLC using a chiral column (Chiralcel OD, Daicel Chemical Industries, Ltd., Japan). The mobile phase was a mixture of *n*-hexane/isopropanol/trifluoroacetic acid (100:1:0.1 v/v/v) at a flow rate of 1.0 mL/min and detection was by UV at 254 nm.

4.4. Enantioselectivity-value measurements

The value of enantioselectivity (*E*-value) was calculated from the enantiomeric excess of the substrate (ee_s) and the conversion degree (*c*) according to the method described by Chen et al.[31] (Eq. 1):

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

Using the equations discussed above, the expected enantiomeric excess of substrate (ee_s) and product can be calculated for a chosen point of conversion and the *E*-value can be determined as a convenient constant value for the selectivity of an enzymatic resolution.

4.5. Statistical analysis

One-way analysis of variance (ANOVA) and Tukey's studentized range test were used to determine the differences in mean *E*-values based on data collected from three replications of each measurement. Significance was established at $p \leqslant 0.05$.

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