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Article

Potential Application of Native Lipases in the Resolution of (*RS*)-Phenylethylamine

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O desempenho de duas lipases nativas (lipase de *Aspergillus niger* e *Rhizopus oligosporus*), e a influência da temperatura, doador acila (acetato de etila, acetato de vinila, acetato de *iso*-propenila e anidrido acético) e do meio orgânico foi avaliado na resolução da (*RS*)-feniletilamina (**1**). O efeito de anions em uma série de líquidos iônicos (LIs) baseados em derivados do imidazol [BMIm][X], onde X = BF_4, PF_6, SCN e Cl em *n*-heptano e acetato de vinila, também foi verificado com a lipase nativa de *A. niger*. Com esta lipase, a amida *R*-**2b** foi obtida com conversões de 6 até > 99% e valores de E de 2 até > 200, em *n*-heptano ou *n*-hexano. Os maiores valores de E obtidos, usando sistema bifásico formado por *n*-heptano e [BMIm][PF_6] ou [BMIm][BF_4] 9:1 (v/v), foram de 9 e 7, respectivamente, quando comparado com o solvente puro (E = 2). A influência dos ânions segue a seguinte ordem: PF_6^- >BF_4^-> SCN^-> Cl^-.

The performance of two native lipases (lipase from *Aspergillus niger* and *Rhizoupus oligosporus*) in the resolution of (*RS*)-phenylethylamine (**1**), varying the temperature, acyl donor type (ethyl acetate, vinyl acetate, *iso*-propenyl acetate and acetic anhydride) and organic medium, was studied. The effect of the nature of the anion using native *A. niger* lipase in *n*-heptane with a series of imidazolium-based ILs [BMIm][X], where X = BF_4, PF_6, SCN and Cl, was also evaluated. Using the lipase from *A. niger*, the *R*-**2b** amide was obtained with conversions from 6 to > 99% and E-values from 2 to > 200, with *n*-heptane or *n*-hexane. This lipase showed better E-values in a two-phase system using *n*-heptane and [BMIm][PF_6] or [BMIm][BF_4] 9:1 (v/v), obtaining values of 9 and 7, respectively, when vinyl acetate was used as the acyl donor, compared to the use of pure *n*-heptane (E = 2). The series for the anions in terms of decreasing performance was as follows: PF_6^- >BF_4^-> SCN^-> Cl^-.

Keywords: native lipases, amine, resolution, ionic liquids

Introduction

Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3) are the most widely applied enzymes in regio-selective and enantioselective biotransformations, since they are inexpensive, stable and easy to recycle. Lipases can be found in many animal and plant tissues.[1-3] They can also be produced by fermentation processes using several microbial species, namely fungi and bacteria. From the economic and industrial standpoints, microorganisms are preferable to animals and plants as enzyme sources. Filamentous fungi are preferred sources of lipases since they tend to produce extracellular enzymes, facilitating extraction from the fermentation growth medium. The most productive species belong to the genera *Rhizopus*, *Rhizomucor*, *Mucor*, *Geotrichum*, *Aspergillus* and *Penicillinium.*[4]

Lipases possess wide substrate specificity and catalyze many reversible reactions in aqueous and non-aqueous media, such as esterification, transesterification, amidation, hydrolysis, epoxidation and dipeptide synthesis.[1,5-10]

Amides are a very important class of organic compounds with a wide range of applications. Some amide derivatives exhibit biological properties such as anthelmintic, antihistamine, antifungal and antibacterial properties.[11,12]

Numerous studies involving enzymatic aminolysis have been reported recently.[7,11-13] The catalytic activity and selectivity of lipases can be improved by changing the solvent, the acyl donor or both.[11,14] The lipase-catalyzed resolution of racemic amines has been successfully carried out in low polarity organic solvents.[15]

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Wen *et al.*[7] reported the aminolytic activity of an immobilized extracellular lipase (LIP2) from *Yarrowia lipolytica* using (±)-α-phenylethylamine with an acetic ester in a medium containing a co-solvent. When the resolution of the (±)-α-phenylethylamine was carried out in a single organic solvent (*viz.* hexane), the enantioselectivity of the lipase was extremely low, but with the addition of 3% (v/v) DMSO to the hexane, the enantiomeric excess of product (ee_p) markedly increased from 0.35 (in pure hexane) to 0.96 and the enantiomeric ratio (E) improved from 2.5 (in pure hexane) to 190. The immobilized lipase could be reused for at least five consecutive batches at high E (≅ 190).[7]

Recently, biocatalysis in non-aqueous media has been extensively used for the resolution of alcohols and amines, but the use of solvents has certain disadvantages, such as being volatile and toxic to the environment, particularly when used on a large scale.[13] The use of ionic liquids (ILs) has emerged contemporarily in organic synthesis, and in some cases can be highly efficient in biocatalysis. These are salts that have an organic cation and an inorganic anion and are often fluid at room temperature with a melting point below 100 °C. Ionic liquids can be used in an enzymatic system in three different ways: as co-solvent in the aqueous phase, as a pure solvent and as a two-phase system together with other solvents. These solvents possess several interesting properties, such as ease of preparation, reuse, high thermal stability and low vapor pressure. They also have widely regulated assets with regard to polarity, hydrophobicity and solvent miscibility behavior, by means of appropriate modification of the cation and anion.[14,15] In addition, the use of ILs in biocatalysis can enhance enzyme stability, substrate and/or product selectivity, and can suppress side reactions.[13,14-17] Although enzymatic transformations in ionic liquids have only been considered since the beginning of the 21st century, a wide number of applications have already been tested in this field, such as esterification, transcyanidation, oxidoreduction, hydrogenation, amidation and acylation,[16,18-22] and ILs have gained wide popularity not only in conventional synthetic chemistry, but also in biotransformation processes.[21]

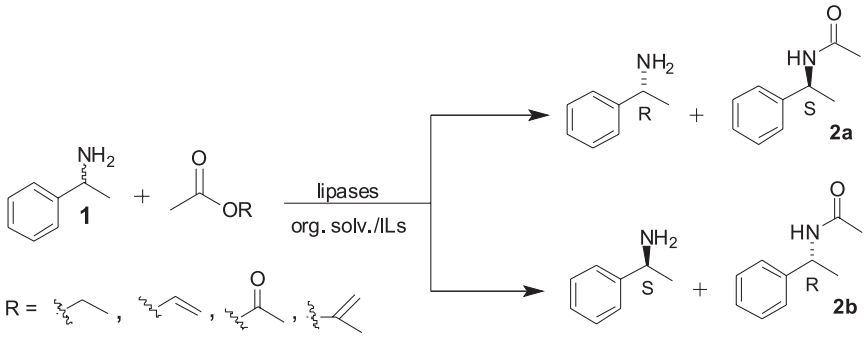
Considering the extraordinary microbial diversity and importance of the fungi as enzyme producers, this paper reports the performance of two native lipases (*Aspergillus niger* and *Rhizopus oligosporus*) in the resolution of (*RS*)-phenylethylamine (**1**). Other parameters included the influence of temperature, acyl donors (ethyl acetate, vinyl acetate, *iso*-propenyl acetate and acetic anhydride) and the organic medium. The effect of anions on the resolution of (*RS*)-**1** using *A. niger* in pure *n*-hexane or *n*-heptane or in mixtures with ionic liquids from a series of imidazolium-based ILs [BMIm][X], where X = BF_4, PF_6, SCN and Cl was also evaluated (Scheme 1).

Experimental

Materials and methods

Lipases from *A. niger* AC-54 (19.4 U mL^{-1}) and *R. oligosporus* (14.9U mL^{-1}) were isolated and purified, and the activity determined as previously described by Carvalho *et al.*[23] and Carvalho and co-workers[4,20] (*RS*)-phenylethylamine was purchased from Sigma-Aldrich. The reagents *n*-hexane, *n*-heptane, ethyl acetate, vinyl acetate, acetic anhydride and *iso*-propenyl acetate were obtained from Vetec (Brazil) and Sigma-Aldrich in the highest purity available. The ionic liquids 1-butyl-3-methyl imidazolium thiocyanide [BMIm][SCN] (≥ 98%), 1-butyl-3-methyl imidazolium chloride [BMIm][Cl] (≥ 98%), 1-butyl-3-methyl imidazolium hexafluorphosphate [BMIm][PF_6](≥ 98%) and 1-butyl-3-methyl imidazolium tetrafluoroborate [BMIm][BF_4] (≥ 97%) were purchased from Sigma-Aldrich or Fluka.

The amount of water in the free enzymes was evaluated by Karl-Fisher titration and they contained 10-12%, which is the amount necessary to preserve the catalytic activity.[24] All other solvents were of analytical grade and were dried by storing over activated 3 Å molecular sieves before use.



Scheme 1. Resolution of (*RS*)-phenylethylamine (**1**) with acyl donors mediated by native lipases.

*General procedure for the lipase-catalyzed resolution of (RS)-**1** with different acyl donors*

A solution of (*RS*)-phenylethylamine (**1**) (2 mmol; 0.26 mL) and ethyl acetate, vinyl acetate, acetic anhydride or *iso*-propenyl acetate (8 mmol) was added to the lipases (50 mg) in *n*-heptane or hexane (25 mL) or in mixtures with ionic liquids (expressed by v/v) at 25-45 °C. In each case, the mixture was shaken on an orbital shaker (100 rpm). The reaction progress and enantiomeric excess values were measured using a gas chromatograph (GC) equipped with a chiral column (CP-chirasil-Dex CB, packed with β-cyclodextrin, 25m × 0.25mm × 0.25mm, Varian). For the analysis, H_2 was used as the carrier gas, the detector and injector were set at 230 °C, and the column was raised from 60 to 200 °C at 3 °C min^{-1}. The retention times of *S*-amide **2a** and *R*-amide **2b** were 24.6 and 25.5 min, respectively, and these data were also compared with enantiopure standard compounds using chiral gas chromatography.

The enantiomeric ratio (E-value) was calculated from the enantiomeric excess of the product (ee_p), and the conversion degree (c) according to the method described by Chen *et al.*[25]

Results and Discussion

Screening of native lipases

In the first stage of this study, two native lipases obtained from *A. niger* and *R. oligosporus* were screened for the resolution of (*RS*)-phenylethylamine (**1**) with ethyl acetate in pure heptane or hexane at 35 °C.

When the lipase from *R. oligosporus* was employed in its native form in heptane or hexane, no product was detected until 144 h of reaction time.

However, better results were obtained when the lipase from *A. niger* in its native form was employed using *n*-hexane or *n*-heptane as the organic solvent, obtaining conversion degrees of 1 and 30%, respectively, with an enantiomeric excess of the product of 27% and > 99%, resulting in E-values of 2.4 and > 200 after 96 h of reaction. Recently, when CAL-B was used as the biocatalyst, similar conversion, ee_p and E-values were obtained for the resolution of (*RS*)-**1**, giving values for these parameters of 23-45%; > 99% and > 200, respectively.[18] These results showed that the lipase from *A. niger* presented good potential for use in this type of reaction. This native lipase was previously used in organic media in the resolution of Ibuprofen.[4,20,26]

Based on these results, *A. niger* lipase was selected as the catalyst for the evaluation of the effect of the organic solvents, and in mixtures of the organic solvents with ionic liquids. Also, the influence of the temperature and acyl donors was evaluated in subsequent experiments.

Effect of reaction temperature

Temperature has a significant influence on the activity, selectivity and stability of a biocatalyst and also on the equilibrium of a reaction. Thus this effect was investigated in the resolution of (*RS*)-**1** at three different temperatures ranging from 25 to 45 °C. The results showed that both the conversions and the enantiomeric ratios were dependent on this parameter (Figure 1).

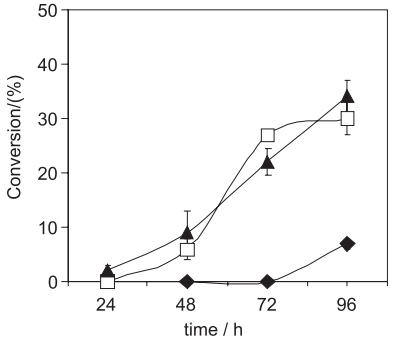


Figure 1. Effect of temperature (°C) on the conversion degree (%) in the resolution of (*RS*)-phenylethylamine (**1**) with ethyl acetate catalyzed by native lipase from *A. niger* (50 mg) in *n*-heptane (25 mL) at (◆) 25 °C, (□) 35 °C, (▲) 45 °C.

The results showed that the conversion degrees increased (from 7 to 34%) as the temperature increased from 25 to 45 °C, after 96 h of reaction. The conversion degrees were similar in the temperature range from 35-45 °C. These results are in agreement with those reported in the literature. Carvalho and co-workers[4] and Carvalho *et al.*[23] reported that the lipases isolated from Brazilian soil samples, such as those from *A. niger*, *Geotrichum candidum* and *Penicillium solitum,* were stable and maintained 100% of activity in the temperature range from 35-45 °C.

At temperatures of 25, 35 and 45 °C, the E-values remained constant (> 200) after 96 h of reaction. Considering the above results, a temperature of 35 °C was selected for use in the study of the influence of the acyl donor and of ionic liquids in the resolution of (*RS*)-**1**.

Influence of the acyl donor

For the resolution of (*RS*)-phenylethylamine (**1**) catalyzed by the lipase from *A. niger* in *n*-heptane, four different acyl donors (ethyl acetate, *iso*-propenyl acetate, vinyl acetate and acetic anhydride) were screened, and the results are presented in Table 1.

Table 1. Effect of the acyl donor in the resolution of (*RS*)-phenylethylamine (**1**) catalyzed by native lipase from *A. niger*

Entry	Acyl donor	time / h	c / (%)	ee_p [a] / (%)	E-value[b]
1	*iso*-propenyl acetate	0.30	74	6	1.2
2	acetic anhydride	0.30	> 99	1	1
3	vinyl acetate	0.30	58	22	2
4	ethyl acetate	48	6	> 99	> 200
5	ethyl acetate	72	27	> 99	> 200
6	ethyl acetate	96	30	> 99	> 200

Reaction conditions: (*RS*)-phenylethylamine (**1**) (2 mmol); acyl donor (8 mmol); *A. niger* (50 mg); *n*-heptane (25 mL), at 35 ºC. [a]Enantiomeric excess of amide **2b**; [b]Enantiomeric ratio.

The conversion degrees were 74%, > 99% and 58% when *iso*-propenyl acetate, acetic anhydride and vinyl acetate were used, respectively. However, the ee_p values and E-values were 6, 1 and 22% and 1.2, 1 and 2, respectively, for 30 min of reaction.

When ethyl acetate was used low conversion degrees were obtained, but the enantiomeric excess of product and E-values were much better. The conversion degrees were from 6-30%, resulting in ee_p values > 99% and E-values > 200 after 96 h of reaction.

In the resolution of (*RS*)-**1**, the native lipase from *A. niger* gave a better result than that obtained in the resolution of (*RS*)-Ibuprofen (E 2.4-6.4).[20] Thus, the choice of a particular substrate structure is also of interest and may influence the E-values of the biocatalytic process.

As observed and discussed, the structure of the acyl donor is crucial. Vinyl acetate, *iso*-propenyl acetate and acetic anhydride proved to be good acyl donors, forming the product with good conversion values, but the E-values were low. Using ethyl acetate, the conversions were moderate but gave high ee_p and E-values.

These results may improve by using ionic liquids (ILs) in mixtures with *n*-heptane, as will be discussed in the following section, and vinyl acetate was selected for this study.

The stereopreference was always towards the (*R*)-enantiomer and these results were verified by comparison with enantiopure samples of *S*- and *R*-amides **2a** and **2b** using chiral gas chromatography.

Effect of ionic liquid

Ionic liquids are increasingly used as reaction media in organic chemistry since they offer a wide range of advantages over classical organic solvents. Basically, the interest in using ionic liquids in biocatalysis is the desire to replace volatile organic solvents by non-volatile ionic liquids.[27] Initially mixtures of *n*-heptane:[BMIm][X] 9:1 (v/v) where X = Cl, SCN, PF_6 and BF_4 were selected, in order to evaluate the influence of the IL and the anions in the enzymatic resolution of (*RS*)-**1** with vinyl acetate.

The four ILs employed for the current study [BMIm][PF_6], [BMIm][BF_4], [BMIm][SCN] and [BMIm][Cl] have distinctly different properties in terms of hydrophobicity, polarity, anion nucleophilicity, hydrogen-bond basicity and viscosity. Since these properties influence the conformation of the lipases and consequently their reactivity, these properties are of primary concern.[7,28]

The results obtained were compared with pure *n*-heptane after 30 min of reaction (Figure 2). For the weakly chaotropic anions Cl^- and SCN^-, the conversion degrees were 67 and 25%, and the ee_p values 3 and 13%, resulting in E-values of 1 and 1.5, respectively.

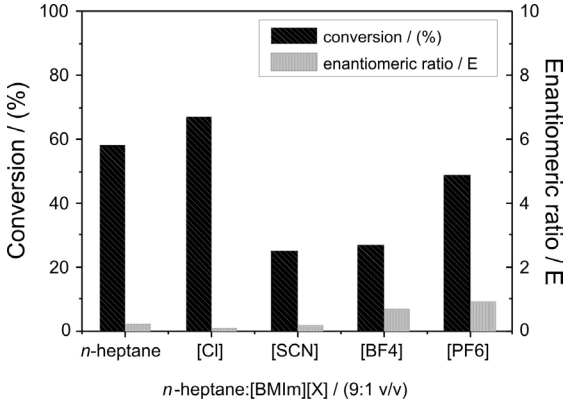


Figure 2. Effect of the anions of the ILs on the resolution of (*RS*)-phenylethylamine (**1**) (2 mmol) with vinyl acetate (8 mmol) using native lipase from *A. niger* (50 mg), *n*-heptane:[BMIm][X] (9:1 v/v) at 35 °C, 30min.

Better results were obtained when the strongly chaotropic anions, BF_4^- and PF_6^- were investigated. The conversion degrees were 27 and 49%, and the ee_p values 62 and 73%, resulting in E-values of 7 and 9, respectively. It should also be noted that the E-value increased from 2 in pure *n*-heptane to 9 using the *n*-heptane:[BMIm][PF_6] mixture. Thus, these data show the dependence of the E-values on the presence of the anions in the ILs. In the reaction studied, the ion effectiveness in enhancing the enzyme selectivity followed the series: $PF_6^- > BF_4^- > SCN^- > Cl^-$ in [BMIm][X]/*n*-heptane mixtures, with vinyl acetate as the acyl donor. The decreasing order of the anion effect was the same as that obtained in the reaction catalyzed by CAL-B in the resolution of (RS)-phenylethylamine, using ethyl acetate in chloroform:[BMIm][X] mixtures.[18]

In the presence of *iso*-octane:[BMIm][PF_6] mixtures in various proportions, using the native lipase from *A. niger* in the resolution of (*RS*)-Ibuprofen, the E-value increased from 2.1 to 4.6.[20]

These studies showed that, in general, enzymes can maintain their activity and stability in certain ionic media. However, not all ILs are suitable for maintaining the activity of an enzyme in a particularly biocatalytic reaction. Thus, there is no simple answer to whether an enzyme is active in a certain IL because the enzyme activity is also dependent on the enzyme-medium-substrate relationship.

Conclusions

In this paper, the resolution of (*RS*)-phenylethylamine (**1**) was studied using two native lipases (*Aspergillus niger* and *Rhizoupus oligosporus*) in pure *n*-hexane or *n*-heptane and in mixtures with ionic liquids from a series of imidazolium-based ILs [BMIm][X], where X = BF_4, PF_6, SCN and Cl. The influence of temperature and the acyl donors was also considered. With respect to conversion, ee_p and E-values, better results were obtained using the lipase from *A. niger* in *n*-heptane and ethyl acetate as the acyl donor at 35 ºC (E > 200). Using *n*-heptane:ILs mixtures with vinyl acetate, the E-values were not very high (E = 1-9) but were better than that obtained with pure *n*-heptane (E = 2). The series for the anions was as follows: $PF_6^- > BF_4^- > SCN^- > Cl^-$.

The results presented in this paper show that the appropriate choice of reaction medium and the nature of the acyl donor appear to be the key to obtaining products with a high enantiomeric ratio and good conversion values, and the search for new biocatalysts with particular characteristics and possible biocatalytic applications is of great interest.

Acknowledgments

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