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Chemo-selectivity of the N,O-enzymatic acylation in organic media and in ionic liquids

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ABSTRACT

The chemo-selectivity and the efficiency of the enzymatic acylation of 6-amino-1-hexanol have been studied in organic solvents distinct by their nature and their dissociation power, in solvent-free systems corresponding to free fatty acid or ethyl ester media and in different ionic liquids. In organic solvents and fatty acid ester media, a sequential reaction allowed the major production of the diacylated derivative at the equilibrium state. Conversely, the use of a solvent-free system with free fatty acid orientated the reaction exclusively towards the O-acylation by modifying the ionization state of the amino group and decreased the reaction time to reach the equilibrium state. Ionic liquids as 1-butyl-3-methyl imidazolium cation coupled with anions of low nucleophilicity significantly improved the efficiency of the reaction (substrate conversion and initial rate) and also led to the N,O-diacyl product. The nature of the reaction medium was shown to influence the ionization state of functional groups, then their capacity to react, and finally, the efficiency of the reaction.

1. Introduction

Acylation reaction provides an excellent method to obtain more apolar derivatives of polar biomolecules. Covalent attachment of fatty acids to proteins or peptides is recognized as a common way for protein/peptide modification which has been shown to influence interaction with membranes [1,2]. For instance, short acylated peptides are able to deeply insert into the hydrophobic core of phospholipids [3]. Generally, peptides are polyfunctional molecules depending on the constitutive amino acids and the major difficulty for the acylation of their nucleophilic sites is the control of the N- and/or O-selectivity.

Different methods of synthesis of such acylated molecules have been developed mainly based on chemical reactions [1,4,5]. These usually lead to the formation of undesirable by-products and mixtures of products with various substitution degrees. For these reasons, biocatalysts with high specificity and selectivity are recognized as appropriate alternatives to catalyze specific acylation reactions of polyfunctional molecules [6]. In the literature, lipases (EC 3.1.1.3) are commonly used to catalyze ester or amide bond synthesis [6–12].

Few studies concern the enzymatic acylation of bifunctional molecules exhibiting both amino and hydroxyl groups in organic solvents as ethanolamine, diethanolamine, serine, and amino alcohols with variable carbon chain length like 3-amino-3-phenylpropanol [7–9,13,14]. In such reactions, the enzyme catalyzed the O-acylation which is followed by a chemical migration of the acyl group from the hydroxyl function to the amine function [13]. The transfer seems to be more difficult when both reactional groups (amine/hydroxyl) are spaced out three carbon or more apart [7–9].

The enzymatic acylation of amino substrate by fatty acid in organic solvent generally leads to the formation of an ion-pair complex between these two molecules depending on the acido-basic conditions of the medium. The latter plays an important role for the transfer of the hydrophilic substrate into the reaction medium [8]. The control of the fatty acid/aminoalcohol molar ratio is essential because it determines the protonation state of ionizable groups of the substrates and their availability.

The solvent-free system is a convenient way to avoid the use of organic solvents which are substituted by an excess of acyl donor. Irimescu et al. [15] demonstrated, for the first time, the synthesis of amide bond under solvent-free conditions by the direct lipase-catalyzed reaction of amines with carboxylic acids for kinetic resolution of primary amines. Their results indicated that the rate of the N-enzymatic acylation in a solvent-free system is reduced due

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to the formation of an unreactive ion-pair complex which limits the availability of the substrate. Tufvesson et al. [16] limited the ion-pair complex formation by adding the amino alcohol progressively and by removing the water formed during the direct acylation. This method mainly orientated the selectivity of the enzymatic acylation towards the amide synthesis.

Ionic liquids have recently emerged to replace organic solvents in biocatalytic transformations especially in the case of polar substrates that are difficult to dissolve in conventional media [17]. These media were used to perform both esters and amides synthesis [12,18,19]. They present unique properties as no vapour pressure, capacity to be recycled and to prevent thermal deactivation of enzymes [20,21]. These media may improve the efficiency of acylation reactions catalyzed by lipases [22–25]. Moreover, they can influence the selectivity for the acylation of polyfunctional substrates in favour of monoacylated products [26]. For instance, a recent study demonstrated that the enzymatic acylation of ethane-1,2-diol in ionic liquids allowed to obtain exclusively the corresponding monoester with a very high yield compared to conventional media [27].

The purpose of the present work was to study the chemoselectivity of the N,O-enzymatic acylation of a bifunctional molecule exhibiting both an amino and a hydroxyl group spaced out of more than three carbons, 6-amino-1-hexanol, by oleic acid or its corresponding ethyl ester. This reaction was catalyzed by an immobilized lipase B of *Candida antarctica* and was firstly performed in organic solvents. The formation of the ion-pair complex between the two substrates was studied and correlated to the chemoselectivity and the efficiency of the reaction (initial rate, reaction duration, yield). Other reaction media like solvent-free system and ionic liquids were used to understand the influence of the reaction environment on performances and selectivity of the reaction.

2. Materials and methods

2.1. Chemicals and enzyme

6-Amino-1-hexanol (97%) and oleic acid (99%) were purchased from Sigma–Aldrich (Steinheim, Germany). Novozym 435® (a lipase B from *Candida antarctica* immobilized on an acrilyc resin) with propyl laurate synthesis activity of 7000 PLU g^{-1} and protein grade of [1–10%] was from Novo Nordisk A/S (Bagsraerd, Danmark). 2-Methyl-2-butanol, hexane, 1-butanol, acetic acid, methanol, chloroform and trifluoroacetic acid (TFA) with 99% of purity were acquired from Carlo Erba (Rodano, Spain). 1-Butyl-3-methylimidazolium hexafluorophosphate ($[Bmim]^+[PF_6]^-$) (99%), 1-butyl-3-methylimidazolium tetrafluoroborate ($[Bmim]^+[BF_4]^-$) (98%) and 1-butyl-3-methylimidazolium dicyanimides ($[Bmim]^+[N(CN)_2]^-$) (98%) were produced by Solvionic SA (Verniole, France). 1-Ethyl-3-methyl-imidazolium-ethylsulfate ($[Emim]^+[EtSO_4]^-$) (98%) and cocosalkyl pentaethoxi-methyl ammonium methosulfate (ECOENG™ 500) (98%) were purchased from Solvent Innovation (Koln, Germany).

2.2. Solubility of 6-amino-1-hexanol

The solubility of 6-amino-1-hexanol in different media was determined at 55 °C as follows: 3 mL of the medium of interest was saturated with the corresponding solid substrate overnight. An aliquot of the supernatant was taken out and filtered then diluted 1000 times with a mixture of methanol/TFA (100/0.1 v/v) and subsequently quantified by HPLC.

The solubility of the substrate in the different reaction media and their respective viscosity were indicated in Table 1.

Table 1
Solubility of 6-amino-1-hexanol at 55 °C, in reaction media used for the enzymatic acylation and their respective viscosity at 25 °C

Reactional media	6-Amino-1-hexanol solubility (g/L)	Viscosity[a] (mPa s)
2-Methyl-2-butanol	188	5.1
Hexane	149	0.3
Oleic acid	276	27.6
Ethyl oleate	288	37.4
$[Bmim]^+[PF_6]^-$	293	287.5
$[Bmim]^+[BF_4]^-$	254	104.2
$[Bmim]^+[N(CN)_2]^-$	173	50.2
$[Emim]^+[EtSO_4]^-$	251	109.2
ECOENG™ 500	123	3000.6

[a] Merck index.

2.3. Acylation procedure

The enzymatic acylation of 6-amino-1-hexanol in ionic liquids, solvent-free systems as well as in organic solvents was carried out in stirred flasks. In a typical acylation reaction, 6-amino-1-hexanol (0.12 M) and oleic acid or ethyl oleate (0.24 M) were added in 1 mL of ionic liquid or organic solvent previously dehydrated on 4 Å molecular sieves. The initial water activity of the media was determined by a thermoconstanter Novasina®, (Switzerland). In all cases, the flasks were incubated in a Syncore® parallel reactors (Büchi) equipped with a condensation system to prevent the evaporation of the solvent. In the case of hexane, an internal chemically inert standard was used (2,6-dimethylphenol at 2 g/L). Reactions were stirred at 250 rpm and heated at 55 °C. After the dissolution of the substrates for 12 h, the acylation of 6-amino-1-hexanol was started by the addition of 10 g/L of lipase preparation. Control experiments without enzyme were also carried out. In the case of organic media, samples were withdrawn and analyzed. Concerning the ionic liquids, various flasks containing the same concentration of reactants and enzyme were prepared and incubated in the same conditions as described before. At different times, the reaction media were withdrawn in order to determine the reaction progress.

Then, the enzyme was removed by filtration and the reaction mixture was diluted with methanol/TFA (100/0.1 v/v). The substrates and the products of the reaction were separated and quantified by HPLC.

To validate the repeatability of the experiments, each reaction was repeated three times. Initial rates and substrate conversion yields are expressed as mean values with standard deviations (±).

2.4. Analytical methods

2.4.1. Qualitative analyses by TLC

Qualitative analyses of the reaction media by TLC were performed on precoated Kieselgel G 60 Plates 20 cm × 20 cm (Merck, Darmstadt, Germany). Two distinct mobile phases were used: butanol (60%)/acetic acid (20%)/water (20%) that permitted to separate with a high resolution 6-amino-1-hexanol from the monoester product (but the more apolar compounds were not separated). Chloroform (95%)/acetic acid (0.5%)/methanol (4.5%) allowed to separate the amide-ester product from the acyl donor.

The molecules exhibiting a primary amino group were detected by spraying an ethanol solution of ninhydrin (0.01%). Other molecules were revealed by spraying an aqueous solution of $KMnO_4$ (0.1 M).

2.4.2. Quantitative analyses by HPLC

The time course of each reaction was monitored using HPLC (LC 10 AD – VP, Shimadzu, France) equipped with an UV detector at 214 nm and a light-scattering low temperature evaporative

Table 2
Partition coefficients of substrates and products in ionic liquid/hexane biphasic systems at room temperature

Ionic liquids	6-Amino-1-hexanol	Oleic acid	Ester product	Amide-ester product
ECOENG™ 500	0.85	0.95	0.89	0.90
[Bmim] + $[PF_6]^-$	0.90	0.99	0.96	0.98
[Bmim] + $[BF_4]^-$	0.73	0.87	0.88	0.87

detector (Shimadzu, France) in this order. The column was a newly developed C18 amide 125–2.1 mm (Altima®, Altech, France) maintained at 25 °C. The mobile phase (0.2 mL/mn flow rate) consisted of methanol/water/TFA (80/20/0.1 v/v/v). A linear elution gradient was applied to reach methanol/TFA (100/0.1 v/v) after 5 min. This methanol concentration was maintained for 18 min and progressively decreased to reach the initial methanol/water ratio until the end of the run (i.e. 34 min). Calibrations were performed using standard substrates and purified products. The substrate conversion rate at the thermodynamic equilibrium was determined applying the following equation:

$$r(\%) = (1 - ([\text{substrate}]_{\text{equilibrium}}/[\text{substrate}]_{\text{initial}})) \times 100.$$

Initial rates of reaction were calculated using a second order polynomial model applied to the derivates of the five first experimental data exhibited in the figures (Software: Matlab®, MathWorks, USA).

When ionic liquids were not miscible to the HPLC mobile phase or interfered with the chromatograms, a preliminary extraction with hexane was applied.

The partition coefficients between ionic liquid and hexane have been determined for all compounds (substrate, acyl donor and acylated products) using the following procedure: a solution of ionic liquids with a known concentration of each compound was prepared and then extracted in hexane (5 v/v). Hexane was evaporated and the residue was solubilized in methanol, and then quantified by HPLC. The partition coefficients were defined as the ratio of final quantity to initial quantity and were mentioned in Table 2. They were used to correct the concentration values.

2.5. Purification of acylated products

2.5.1. O-oleylaminohexanol

After filtration of the reaction medium to remove the enzyme and partial evaporation of the solvent, the residue was applied to a silica gel column (Silica Gel 60, 230–400 mesh-Merck, Darmstadt, Germany) and eluted with butanol/acetic acid/water (60/20/20 v/v/v). Fractions were collected and then analyzed by TLC (precoated Kieselgel 60 Plate 20 cm × 20 cm (Merck). The fractions containing the O-oleylproduct were washed with water until neutrality. To favour the neutral form of the amino group, a last wash with an alkaline aqueous solution (pH = 13) was carried out. The solvent was evaporated under reduced pressure.

2.5.2. N,O-dioleylaminohexanol

After elimination of the enzyme, the reaction medium was concentrated by the partial evaporation of the solvent and then applied to a preparative TLC plate coated with Kieselgel 60. The mixture was developed with chloroform/acetic acid/methanol (95/0.5/4.5 v/v/v). A short portion of the migration band containing the N,O-dioleylaminohexanol was detected by spraying a $KMnO_4$ solution. The silica was collected and the amide-ester was solubilized in methanol. Silica was eliminated by filtration and methanol was evaporated under reduced pressure.

2.6. Identification of purified products

2.6.1. Electrospray ionization mass spectrometry analysis

The mass of the monoacylated product was verified on a SCIEX API 150 EX mass spectrometer with electron spray ionization source. MS analysis was carried out with helium as the collision gas. MS parameters were tuned as following: positive and negative ionization mode, capillary temperature of 150 °C, source voltage at 5.0 kV. The positive ion ESI mass spectra were obtained by spraying the sample in methanol/water (1/1), at a flow rate of 5 μL/min. Mass spectra were acquired by scanning a m/z range from 100 to 2000. O-oleylaminohexanol, MS spectra; $m/z = 382\ \text{g mol}^{-1}$ ($M+H^+$); $m/z = 405\ \text{g mol}^{-1}$ ($M+Na^+$).

2.6.2. Nuclear magnetic resonance

The chemical structure of the purified acylated products was determined by ^{13}C NMR and ^{1}H NMR spectroscopic analysis in $CDCl_3$ on a Brücker, 300 MHz, 7.1 T spectrometer (Germany), m: multiplet, br t: broad triplet. O-oleylaminohexanol: ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 0.88 (*t*, $J = 6.6$ Hz, 3H, $-CH_3$), 1.50 (m, $-CH_2-$ of 6-amino-1-hexanol), 2.30 (*t*, $J = 7.25$ Hz, 2H, $-CH_2-CO-O-$), 3.00 (br *t*, 2H, $-CH_2-NH_2$), 4.05 (*t*, $J = 6.65$ Hz, 2H, $-CH_2-O-CO-$), 5.34 (m, 2H, $-CH=CH-$). ^{13}C NMR (300 MHz, $CDCl_3$) δ ppm: 14.77 (1C, $-CH_3$), 23.35 (1C, CH_3-CH_2-), 25.65–32.58 (multiple pics, $-CH_2-$ of oleyl chain), 35.02 (1C, $-CH_2-CO-O-$), 40.57 (1C, $-CH_2-NH_2$), 64.63 (1C, $-CH_2-O-CO-$), 130.68 (2C, $-C=C-$), 174.69 (1C, $-CO-O-$).

N,O-dioleylaminohexanol: ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 0.81 (*t*, $J = 6.6$ Hz, 6H, 2× CH_3), 1.29 (m, $-CH_2-$), 1.48 (m, $-CH_2-$), 1.75 (m, 8H, 2× $-CH_2-C=C-CH_2-$), 1.93 (*t*, $J = 7.26$ Hz, 2H, $-CH_2-CO-NH-$), 2.10 (*t*, $J = 7.25$ Hz, 2H, $CH_2-CO-O-$). 3.19 (m, 2H, $-CH_2-N-CO-$), 4.24 (*t*, $J = 6.65$ Hz, 2H, $-CH_2-O-CO-$), 5.27 (m, 4H, 2× $-CH=CH-$), 5.43 (br *t*, 1H, $-NH-CO-$). ^{13}C NMR (300 MHz, $CDCl_3$) δ ppm: 14.79 (2C, $-CH_3$), 23.37 (2C, $-CH_2-CH_3$), 25.94–33.19 (multiple pics, $-CH_2-$), 35.09 (1C, $-CH_2-CO-O-$), 37.45 (1C, $-CH_2-CO-NH-$), 40.10 (1C, $-CH_2-NH-CO-$), 63.36 (1C, $-CH_2-O-CO-$), 130.57 (4C, $-CH=CH-$), 174.2 (2C, $-CO-O-$ and $-CO-NH-$).

2.7. Study of the ionization state of the substrates in organic solvents

The ionization state of 6-amino-1-hexanol and oleic acid during the acylation in 2-methyl-2-butanol was investigated by infrared spectroscopy (FTIR). The infrared (IR) spectra of samples were recorded from 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1} using a Brücker TENSOR 27 spectrometer and a total attenuated reflexion cell thermostated at 55 °C. Before the interpretation of data, a treatment (base line correction, smoothing and normalization min–max) was applied to the spectra.

3. Results and discussion

6-Amino-1-hexanol is a bifunctional molecule with a hydroxyl group (primary alcohol) and an amino group (primary amine). Acy-

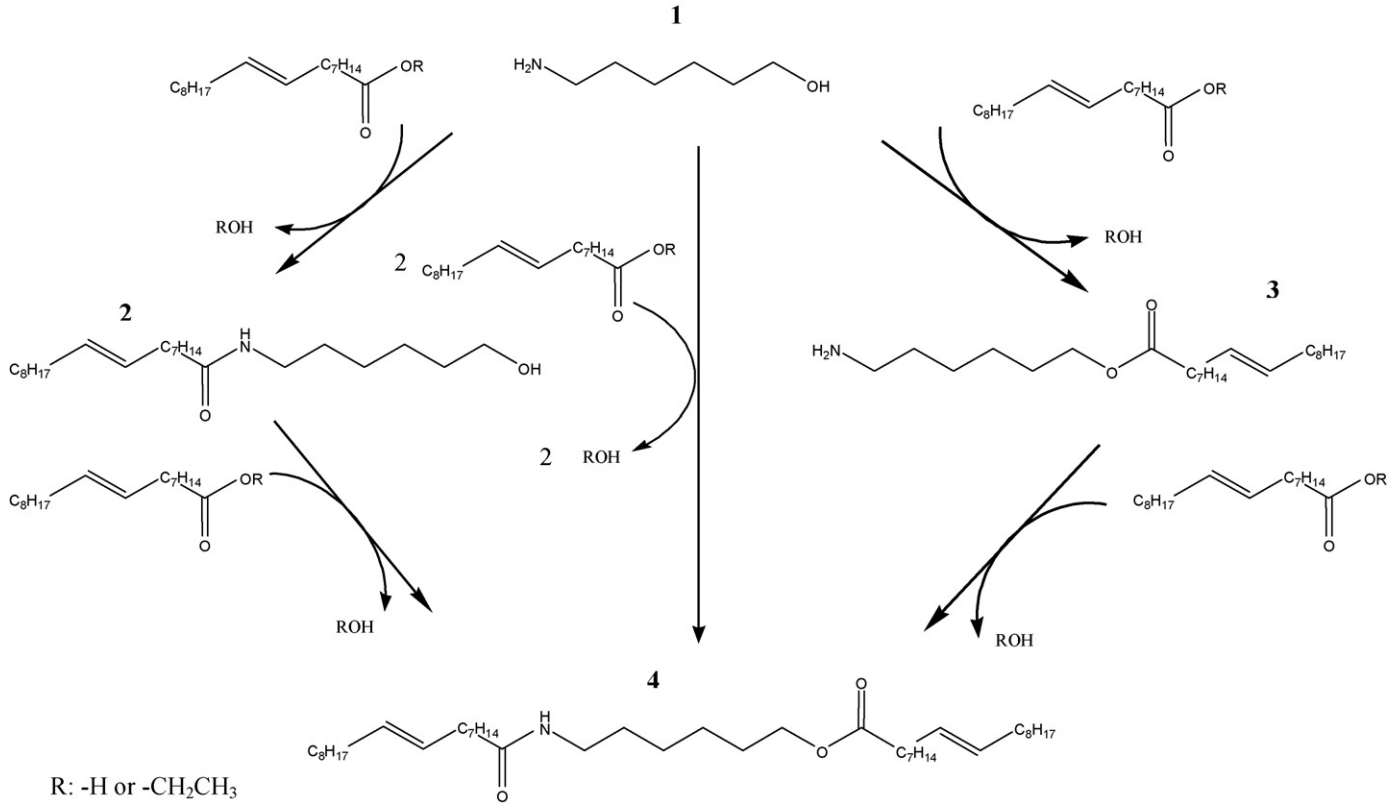

Fig. 1. Acylation possibilities of 6-amino-1-hexanol. **1**: 6-Amino-1-hexanol, **2**: N-oleylaminohexanol, **3**: O-oleyl product, **4**: N,O-dioleylaminohexanol.

lation possibilities of this substrate by oleic acid or ethyl oleate are presented in Fig. 1.

According to previous works using Novozym 435® in a wide range of temperature, enzymatic acylation reactions were carried out at 55 °C [7,8,28]. As an excess of acyl donor appeared to improve the catalytic activity, the molar ratio acyl donor to acceptor in this study was set to 2 [29].

No product was obtained in media containing 6-amino-1-hexanol and the acyl donor (oleic acid and ethyl oleate) at 55 °C, in absence of enzyme.

3.1. N,O-enzymatic acylation of 6-amino-1-hexanol in organic solvents

The N,O-enzymatic acylation of 6-amino-1-hexanol was studied in two organic solvents which exhibited different physicochemical properties: the 2-methyl-2-butanol is a protic and polar solvent with a log*P* value of 1.31 and a dielectric constant of 32.6 μS cm^{-1}. Hexane is an aprotic and apolar solvent (log*P* = 3.4 and ε = 1.89 μS cm^{-1}). The solubility of 6-amino-1-hexanol in 2-methyl-2-butanol and in hexane at 55 °C is indicated in Table 1 and showed that at 0.12 M, the substrate is totally solubilized in both solvents. According to the distinct physicochemical properties of these two solvents, the objectives were to study the ionization state of the substrates in these two reaction media, then its influence on the efficiency and the selectivity of the enzymatic acylation.

3.1.1. Qualitative analyses of the reaction media

Several studies reported that in apolar media, the ionization state of substrates and the ionization state of the enzyme microenvironment can play an important role in reactions catalyzed by the immobilized lipase B of *Candida Antarctica*. Maugard et al. [30] showed that the formation of an ion-pair complex was important to favour enzymatic acylation especially in the case of insoluble substrate. However, when this ion-pair complex was less soluble than the free substrate, its presence constituted the limiting factor of the reaction.

To verify the presence of this ion-pair complex in both solvents, FTIR analyses were performed during the course of 6-amino-1-hexanol acylation.

The IR spectrum of oleic acid solubilized in 2-methyl-2-butanol or in hexane at 55 °C exhibited a band at 1710 cm^{-1} which is specific of carboxylic acid function. When 6-amino-1-hexanol was added in the medium, this band diminished and a new band was detected at 1566 cm^{-1} corresponding to the carboxylate ion. This observation demonstrated that an ion-pair complex was formed when both substrates are mixed in the media as already observed in other studies [29,30]. Before the addition of the enzyme, the majority of the substrates was under soluble ionic complex form. During the reaction the bands at 1566 cm^{-1} and at 1710 cm^{-1} diminished and a band at 1660 cm^{-1} appeared which is specific of the amide bond. In the present study, the free 6-amino-1-hexanol was totally soluble in both organic solvents and the results suggested that the soluble ionic complex did not limit the synthesis of acylated products. The availability of the amino substrate was probably influenced by the nature of the solvent, especially by its dissociating efficiency.

The time course of the enzymatic acylation of 6-amino-1-hexanol in 2-methyl-2-butanol and in hexane was preliminary analyzed by TLC. This qualitative approach showed the rapid transformation of 6-amino-1-hexanol in favour of the synthesis of two more hydrophobic products. The first one exhibited a free amino group and was rapidly converted into a more apolar product without any free amino group. These results indicated the sequential synthesis of two products which can result from the O-acylation of 6-amino-1-hexanol, then the N-acylation of the O-acyl product (Fig. 1: molecules 3 and 4).

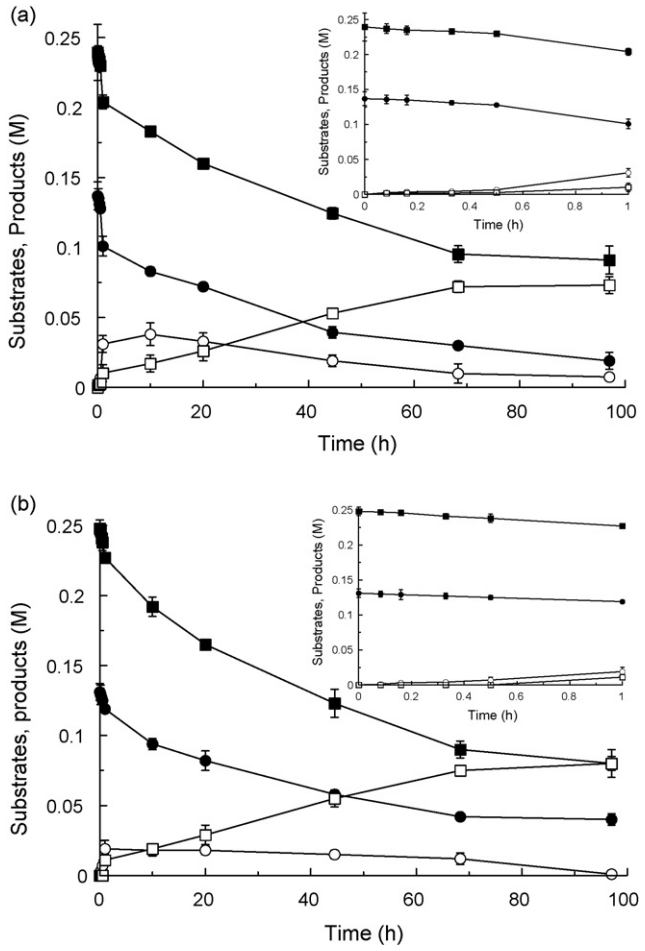

Fig. 2. Kinetic curves related to the enzymatic acylation of 6-amino-1-hexanol (0.12 M) by oleic acid (0.24 M) in 2-methyl-2-butanol (a) and in hexane (b). Reactions were carried out at 55 °C and catalyzed by Novozym 435®. (●) 6-Amino-1-hexanol, (■) oleic acid, (○) O-oleylaminohexanol, (□) N,O-dioleylaminohexanol.

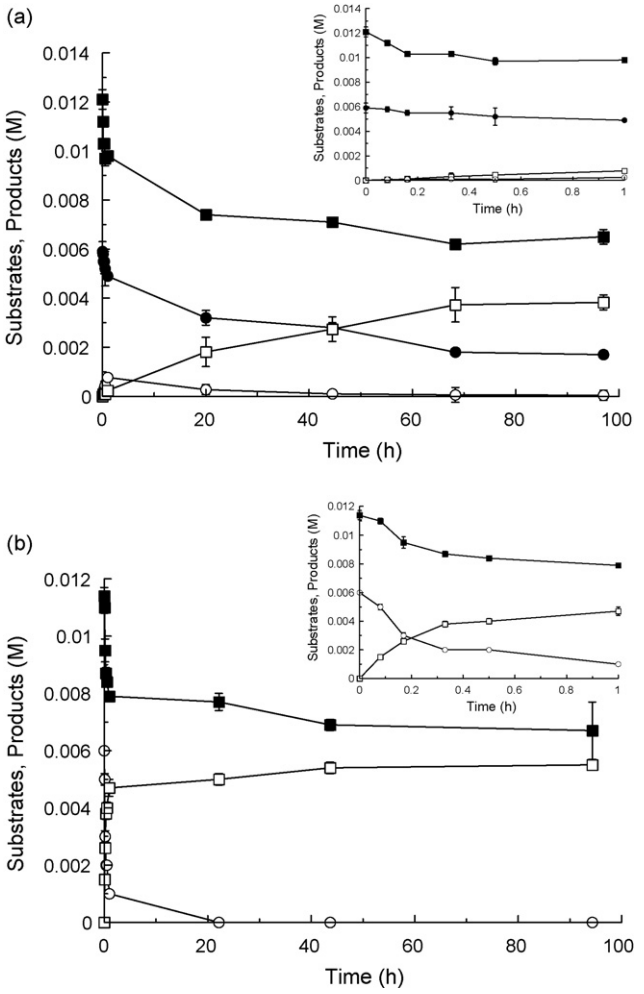

Fig. 3. Kinetic curves related to the enzymatic acylation of 6-amino-1-hexanol (0.006 M) by oleic acid (0.012 M) (a) and the enzymatic acylation of O-oleylaminohexanol (0.006 M) by oleic acid (0.012 M) (b) in 2-methyl-2-butanol. Reactions were carried out at 55 °C and catalyzed by Novozym 435®. (●) 6-Amino-1-hexanol, (■) oleic acid, (○) O-oleylaminohexanol, (□) N,O-dioleylaminohexanol.

The purification and the structural elucidation by mass spectroscopy and NMR analyses confirmed the identity of these products.

3.1.2. Quantitative analysis of the reaction media

The kinetic curves relative to the acylation reaction in organic solvents showed that the conversion of 6-amino-1-hexanol, after 70 h of reaction, was 86 ± 5% and 69 ± 3% in 2-methyl-2-butanol and in hexane, respectively (Fig. 2a and b). At the thermodynamic equilibrium, the major product was the N,O-diacyl product. The initial rates of synthesis demonstrated that the O-acyl product was firstly synthesized and accumulated with an initial rate of $14 \pm 3\ mM\ h^{-1}$ in 2-methyl-2-butanol and $10 \pm 5\ mM\ h^{-1}$ in hexane, then followed by the N,O-diacyl product ($7 \pm 1\ mM\ h^{-1}$ in 2-methyl-2-butanol versus $1 \pm 0.8\ mM\ h^{-1}$ in hexane). The amide product was never detected (Fig. 1: molecule 2). The kinetic profiles showed that the selectivity of the reaction observed in 2-methyl-2-butanol was similar than in hexane. The less conversion of 6-amino-1-hexanol in hexane may be explained by the low dissociating effect of this solvent leading to the increase of the ion-pair complex stability and consequently the decrease of the 6-amino-1-hexanol availability.

Other studies reported that during the enzymatic acylation of short bifunctional molecules, the O-acyl product was never detected [7–9]. This result was attributed to the migration of the acyl residue between the amino and hydroxyl groups. 6-Amino-1-hexanol structure constituted by two functional groups distant of 6 carbons prevents the acyl-transfer explaining the absence of the amide product [13]. Another explanation can be that the N-acylated product was synthesized but immediately converted into the N,O-diacyl product.

O-oleyl-aminohexanol was purified and was shown to be stable. This study demonstrated that Novozym 435® exhibited a reaction selectivity favouring the O-acylation in comparison with the N-acylation. This result is in agreement with other studies [8].

In order to study the specificity of the enzyme forward the O-acylated product in comparison with 6-amino-1-hexanol, the enzymatic acylation of O-oleyl-aminohexanol (6 mM) by oleic acid (12 mM) catalyzed by Novozym 435® was carried out. An enzymatic acylation using 6-amino-1-hexanol as substrate in the same conditions was performed as a reference. The kinetic curves of these two reactions showed a higher initial rate ($13 \pm 2\ mM\ h^{-1}$, Fig. 3a) for the O-oleyl-aminohexanol transformation in comparison with that of 6-amino-1-hexanol ($2 \pm 1\ mM\ h^{-1}$, Fig. 3b). This study demonstrated that during the course of the enzymatic acylation of 6-amino-1-hexanol, a competition between the initial substrate and the O-acyl product occurred suggesting that the lipase B of *C. antarctica* exhibited a higher specificity for the O-acyl substrate in comparison with the unacylated molecule.

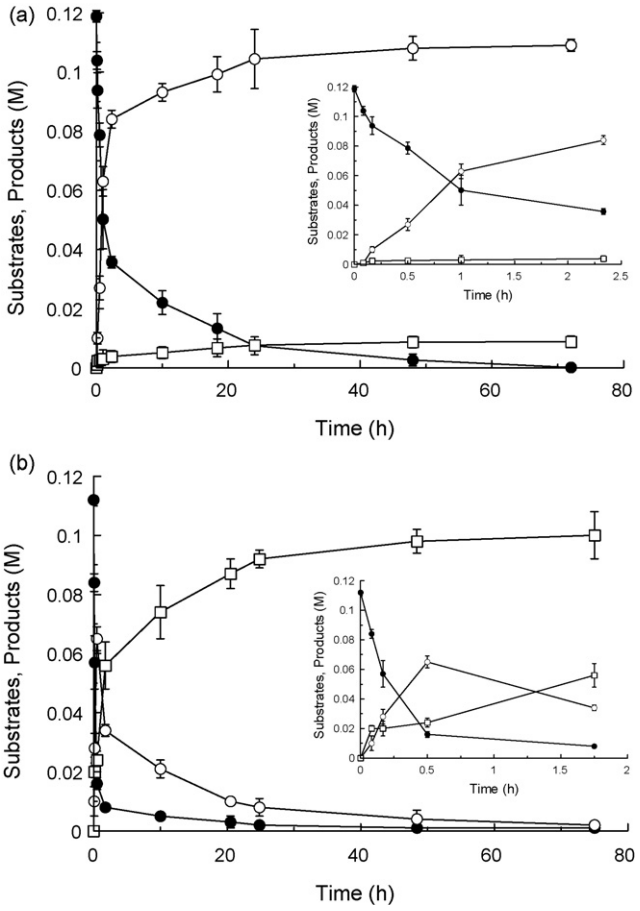

Fig. 4. Kinetic curves related to the enzymatic acylation of 6-amino-1-hexanol (0.12 M) by oleic acid (a) and ethyl oleate (b) in a solvent-free system. Reactions were carried out at 55 °C and catalyzed by Novozym 435®. (●) 6-Amino-1-hexanol, (○) O-oleylaminohexanol, (□) N,O-dioleylaminohexanol.

3.2. N,O-enzymatic acylation of 6-amino-1-hexanol in solvent-free system

In such media, the acyl donor was introduced in large excess and replaced the solvent. These conditions are expected to favour the displacement of the equilibrium towards the synthesis. The N,O-enzymatic acylation of 6-amino-1-hexanol in oleic acid and in ethyl oleate was carried out. At 55 °C, the total solubilisation of 6-amino-1-hexanol in oleic acid and in ethyl oleate (Table 1) was observed (0.12 M). The initial a_w was inferior to 0.1.

Fig. 4a showed that the thermodynamic equilibrium of the acylation in oleic acid was reached in 20 h only. The initial rates of the ester and the diacyl product syntheses (64 ± 2 mM h^{-1} and 11 ± 1 mM h^{-1}, respectively) were much higher than those obtained in organic media. The conversion of the substrate was close to 100%. Conversely to the organic solvents, the O-acyl product was slightly transformed and only few N,O-diacyl product was detected. This result was probably due to the acidity of the medium leading to the protonation of the amino group which lost its nucleophilic property. Furthermore, the excess of fatty acid may enhance the stability of the ion-pair complex. For this reason, these reaction conditions allowed the chemo-selectivity in favour of the O-acylation, while the use of organic solvents led to the major N,O-diacyl product.

To verify the influence of the protonation state of the substrate on the selectivity, the N,O-enzymatic acylation of 6-amino-1-hexanol was carried out in ethyl oleate. In this case, the acyl donor was not able to protonate the amino function of the substrate. Fig. 4b showed that the major product obtained at the equilibrium (reached in 50 h) was the N,O-diacylated product (initial rate of synthesis: 48 mM h^{-1}). The absence of chemo-selectivity was also observed as in organic solvents. These kinetic profiles suggested that in ethyl oleate, the amino group was not majorly protonated and the *trans*-amidification of the O-acyl product was possible.

In conclusion, using solvent-free systems, it was possible to direct the enzymatic acylation of 6-amino-1-hexanol either towards the O-acylation or the N,O-acylation depending on the nature of the acyl donor (free fatty acid or ester).

3.3. N,O-enzymatic acylation of 6-amino-1-hexanol in ionic liquids

In the present work, five different ionic liquids were used to investigate their ability to improve the N,O-enzymatic acylation of 6-amino-1-hexanol and compare the selectivity with that obtained in organic solvents.

Table 1 showed that, for all ionic liquids used, the solubility of 6-amino-1-hexanol (0.12 M or 14 g/L) was total.

3.3.1. Ionic liquids with constitutive sulphate anion

Table 3 presented the substrate conversion obtained at the equilibrium in the five ionic liquids tested. For the ionic liquids [Emim]$^+$[EtSO$_4$]$^-$ and ECOENG^{TM} 500, the substrate conversion values were significantly less important than in organic solvents. This result cannot be explained by a limited solubilisation of the substrate.

The acylation reaction in [Emim]$^+$[EtSO$_4$]$^-$ led to a very poor production of the O-acyl-product (inferior to 1 mM) and the amide-ester was not detected. Previous studies about O-acylation reactions in [Emim]$^+$[EtSO$_4$]$^-$ demonstrated that the activity of Novozym 435® in this medium was low. It was suggested that the coordination of [EtSO$_4$]$^-$ anions to the enzyme surface can cause conformational changes leading to a loss of activity [25].

Another ionic liquid with an identical constitutive anion was used, ECOENG^{TM} 500. A substrate conversion of 38 ± 4% was obtained and the ester was the major product at the equilibrium (28 ± 3 mM). This low efficiency can be explained by the high viscosity of this medium that can limit the diffusion of substrates.

Table 3
Efficiency and kinetic parameters of the O and/or N,O-acylation of 6-amino-1-hexanol (0.12 M) by oleic acid (0.24 M) in different ionic liquids and their respective a_w

Ionic liquids	a_w	[Ester product] (mM)	[Amide ester product] (mM)	Initial rates (mM h^{-1})		Substrate conversion (%)
				Ester synthesis	Amide-ester synthesis	
[Emim]$^+$[EtSO$_4$]$^-$	0.04	<1	nd	nd	nd	<1
ECOENG^{TM} 500	0.10	28 ± 3	18 ± 2	nd	nd	38 ± 4
[Bmim]$^+$[N(CN)$_2$]$^-$	0.14	<1	11 ± 3	nd	nd	9 ± 1
[Bmim]$^+$[BF$_4$]$^-$	0.11	12 ± 3	78 ± 1	93 ± 8	48 ± 7	92 ± 2
[Bmim]$^+$[PF$_6$]$^-$	0.09	3 ± 1	109 ± 2	190 ± 20	110 ± 30	97 ± 2

Concentrations of products and substrate conversion were determined at the thermodynamic equilibrium.

Table 4
Selectivity and efficiency for the O and/or N,O-acylation of 6-amino-1-hexanol (0.12 M) by oleic acid (0.24 M) in different media

Reaction media	Selectivity (%)		Reaction duration (h)	6-Amino-1-hexanol conversion (%)
	O-acylation	N,O-acylation		
2-Methyl-2-butanol	7.9	92.1	70	86 ± 5
Hexane	1.2	98.8	70	69 ± 3
Solvent-free system (oleic acid)	91.5	8.5	25	99 ± 1
Solvent-free system (ethyl oleate)	2.5	97.5	50	98 ± 2
$[Bmim]^+[BF_4]^-$	13.3	86.7	25	92 ± 2
$[Bmim]^+[PF_6]^-$	2.7	97.3	25	97 ± 2

Reactions were carried out in Syncore® reactors at 55 °C and catalyzed by Novozym 435®. Reaction duration and substrate conversion corresponded to the equilibrium state.

3.3.2. Ionic liquids with constitutive 1-butyl-3-methylimidazolium cation

Three ionic liquids based on the same constitutive cation: 1-butyl-3-methylimidazolium combined with $[BF_4]^-$, $[PF_6]^-$ or $[N(CN)_2]^-$ were compared.

The use of the ionic liquid dicyanimide led to a very low substrate conversion (9 ± 1%) which can be due to the hydrophilic character of this solvent. This result can be due to the difficulty to reduce the a_w of the medium which still remained at 0.14 after desiccation (Table 3).

For both ionic liquids with $[BF_4]^-$ or $[PF_6]^-$, an excellent substrate conversion (superior to 90%) was obtained after 25 h of reaction (Fig. 5) in comparison with 70 h in organic solvents. All the initial rates of product synthesis were higher than in organic solvents (Table 3). Fig. 5a and b indicated that the O-acyl product was firstly synthesized followed by the N,O-diacyl product in both ionic liquids. These results showed the absence of chemo-selectivity in ionic liquids.

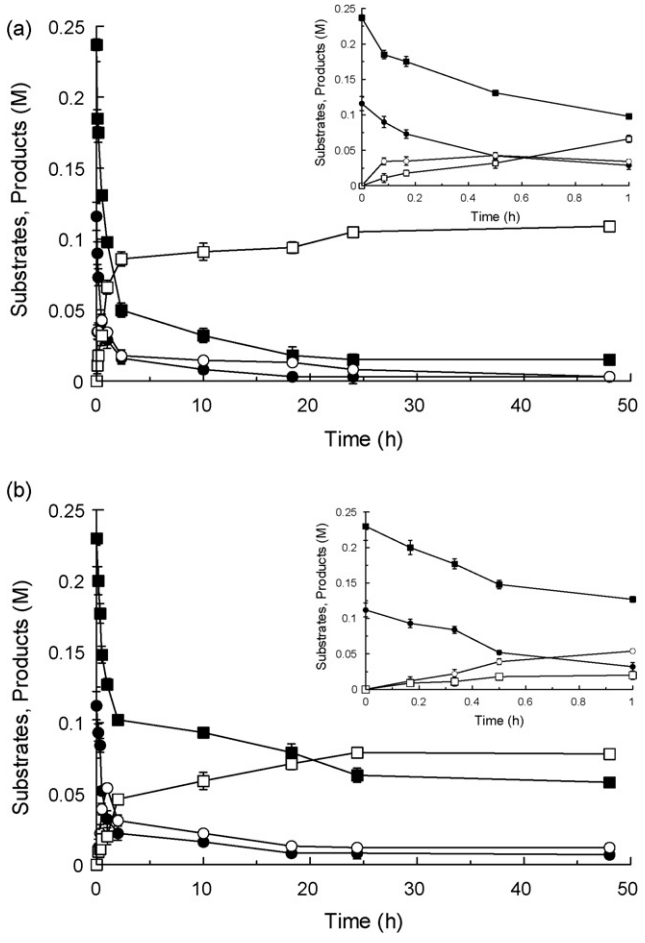

Fig. 5. Kinetic curves related to the enzymatic acylation of 6-amino-1-hexanol (0.12 M) by oleic acid (0.24 M) in $[Bmim]^+[PF_6]^-$ (a) and in $[Bmim]^+[BF_4]^-$ (b). Reactions were carried out at 55 °C and catalyzed by Novozym 435®. (●) 6-amino-1-hexanol, (■) oleic acid, (○) O-oleylaminohexanol, (□) N,O-dioleylaminohexanol.

Moreover the efficiency of the N,O-enzymatic acylation was improved in these ionic liquids. This could be explained by the high polarity of such media which favoured the dissociation of the ion-pair complex. The constitutive cation can establish hydrogen bonds with the amino substrate, resulting in the availability of the acyl donor in excess as suggested by Irimescu et al. [19]. The hydrophilic character of $[Bmim]^+[BF_4]^-$ did not affect the efficiency of the acylation of 6-amino-1-hexanol as demonstrated by Irimescu et al. in the case of primary amine acylation [19].

The nucleophilicity of the constitutive anions probably played a preponderant role in the improvement of the efficiency of the N,O-enzymatic acylation [19]. The efficiency of the N,O-enzymatic acylation was higher in ionic liquids with 1-butyl-3-methylimidazolium cation coupled with anions of low nucleophilicity (Table 3). These results confirmed that the anions exhibiting lower nucleophilicity would not interact with charged sites of enzyme which maintained its optimal activity [25].

4. Conclusion

This study showed that an appropriate choice of the reaction systems (solvent, acylating agent) may allow the chemo-selective acylation of bifunctional molecule as 6-amino-1-hexanol catalyzed by the immobilized lipase B of *Candida antarctica* (Table 4).

At equilibrium, in 2-methyl-2-butanol and in hexane, the substrate conversion was close to 90% and 70%, respectively. The N,O-diacyl compound was obtained majorly in these reaction media (>90%). The use of a solvent-free system significantly decreased the time to attempt the equilibrium. Free fatty acid medium permitted to orientate the selectivity of the reaction towards the O-acylation exclusively (91.5%) due to the ionization state of the amine function. Ethyl ester medium allowed to restore the major synthesis of the N,O-diacyl product.

Finally, the use of ionic liquids like $[Bmim]^+[BF_4]^-$ and $[Bmim]^+[PF_6]^-$ significantly improved the efficiency of the reaction. The substrate conversions were close to 100% in $[Bmim]^+[PF_6]^-$ and 90% in $[Bmim]^+[BF_4]^-$. The reaction duration was significantly decreased and the absence of chemo-selectivity observed in organic solvents was maintained in these ionic liquids. However, these different media did not allow the selective synthesis of the *N*-acyl product.

Ionic liquids appeared to be effective reaction media for acylation reaction catalyzed by the immobilized lipase B of *Candida antarctica*. However, their toxicology remained unclear and cell death through apoptosis has been already suggested in the case of some of them [31]. Supercritical carbon dioxide can be another attractive alternative to perform acylation reaction [32]. Further studies are in progress to focus on peptide acylation to find con-

ditions for the regioselective acylation of amino acids in different media, including ionic liquids and supercritical carbon dioxide.

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