



Kinetic study of 2-butanol O-acylation and sec-butylamine N-acylation catalyzed by *Candida antarctica* lipase B

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ABSTRACT

The aim of this work was to study the differential behavior shown by *Candida antarctica* lipase B during the O-acylation and N-acylation of monofunctional alcohols and monofunctional amines. To achieve this, 2-butanol and sec-butylamine were used as model molecules. Yields, kinetics and enantioselectivity were studied for both reactions. Although a steady-state ordered ternary complex bi–bi mechanism was obtained for the O-acylation of 2-butanol, a ping–pong bi–bi mechanism was obtained for the N-acylation in case of low sec-butylamine concentrations. The values of apparent kinetic parameters were calculated: the enantiomeric ratios (E) were evaluated and confirmed the preference of *C. antarctica* lipase B for the (R)-enantiomer, which was consistent with the literature. The enantioselectivity was calculated for the alcohol ($E \approx 3.17$) and for the amine ($E \approx 1.34$). Concerning the O-acylation, the yields were found to be very similar for both enantiomers R and S. However, both initial rates and yields of the (R)-enantiomer N-acylation were higher than those of the (S)-enantiomer. In the last part of our study, the chemoselectivity of *C. antarctica* lipase B was evaluated, showing that *C. antarctica* lipase B was a chemoselective enzyme that preferentially catalyzed the O-acylation to the detriment of the N-acylation ($C \approx 92$, for the selective acylation of (R)-enantiomers). These results provide new insights for the synthesis of products issued from the selective acylation of multifunctional substrates such as amino-alcohols.

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1. Introduction

Many molecules such as amino acid esters [1], glucamides [2] or ceramides [3] are derived from the selective acylation of multifunctional compounds like amino-alcohols. The method used for the chemical synthesis of these molecules is well established but is confronted to several limitations. It requires fastidious steps of hydroxyl group protection and deprotection for the control of chemoselectivity and stereoselectivity. The high temperatures required also preclude the use of fragile molecules and may cause coloration of final products. In addition, the coproduction of salts, and the use of toxic solvents (dimethylformamide, methanol, etc.) that must be eliminated at the end of the reaction increase the cost of the processes. For these reasons, an interesting alternative is the use of biocatalysts which offers a clean way to perform chemical processes, under mild reaction conditions and with a high degree of selectivity.

Enzymes have been used mostly for aqueous phase reactions. However, non-aqueous enzymology has potential applications in industry. The use of immobilized enzymes, in particular lipases,

in organic media rather than aqueous media has several advantages such as the shift in thermodynamic equilibrium in favor of the synthesis over the hydrolysis reaction, the increased solubility of non-polar substrates, the elimination of side reactions, the ease of enzyme and product recovery and the increased enzyme thermostability.

Lipases can be used to catalyze a wide range of valuable synthesis reactions among which the acylation of primary alcohols and amines. Many models concerning the lipase-catalyzed acylation of primary alcohols in organic solvents or solvent-free systems have already been characterized and shown to kinetically proceed via a ping–pong bi–bi mechanism or sometimes an ordered bi–bi mechanism [4–6]. In some cases, substrate inhibition was observed. For instance, an ordered bi–bi mechanism with inhibition by both substrates was used to model the esterification of cetyl alcohol with oleic acid [7] and a ping–pong bi–bi mechanism implying a competitive inhibition by substrates was described for the transesterification of isoamyl alcohol with ethyl acetate conducted in *n*-hexane as a solvent [8]. A ping–pong bi–bi mechanism with inhibition by the amine was also reported for the N-acylation of ammonia with oleic acid [9]. On the other hand, Arcos et al. did not identify any inhibition step when they proposed a ping–pong bi–bi mechanism to describe the lipase-catalyzed esterification of glucose with fatty acids [10].

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Among lipases used in synthesis, *Candida antarctica* lipase B is well known for its ability to convert alcohols and amines into esters and amides [2,11]. Both alcohols and amines are nucleophiles that can play the role of acyl acceptor. However there is some difference between those two chemical groups that affects the behavior of *C. antarctica* lipase B toward O-acylation and N-acylation. For example, amines are more nucleophilic than alcohols, have a larger steric hindrance that may interfere with their positioning in the active site, have ability to realize more hydrogen bonds, etc. Thus, understanding the kinetic mechanism and selectivity of the O-acylation or N-acylation of monofunctional alcohols or amines catalyzed by *C. antarctica* lipase B is necessary before extending such enzymatic processes to multifunctional molecules.

In this context, the behavior of *C. antarctica* lipase B toward the acylation of monofunctional amines and alcohols was studied in this work (Scheme 1). The kinetic mechanism and the enantioselectivity of the reaction were established for both substrates. Finally, the chemoselectivity of *C. antarctica* lipase B toward the O-acylation and N-acylation was evaluated.

2. Materials and methods

2.1. Enzyme and chemicals

Novozym® 435 (immobilized *C. antarctica* lipase B), was kindly provided by Novozymes A/S, Bagsvaerd, Denmark. R and S pure enantiomers (99%) of 2-butanol and *sec*-butylamine, as well as *tert*-amyl alcohol were purchased from Sigma–Aldrich (St Louis, USA) while myristic acid and acetic acid were from Fluka (St Quentin-Fallavier, Switzerland). All chemicals were dried over molecular sieves. Pure water was obtained via a Milli-Q system (Millipore, France). Acetonitrile was purchased from Carlo ERBA (Val-de-Reuil, France).

2.2. Enzymatic reactions

Initial rate measurements were performed at 55 °C in *tert*-amyl alcohol according to a previously established procedure [12]. 2 ml of the reaction mixtures containing various amounts of substrates (25–350 mM) were incubated for 10 min prior to addition of 10 g l⁻¹ of *C. antarctica* lipase B for the acylation of 2-butanol with myristic acid and 50 g l⁻¹ of *C. antarctica* lipase B for the acylation of *sec*-butylamine with myristic acid. 100 µl samples were taken at intervals and centrifuged at 14,000 rpm. The supernatant was analyzed by LC–MS. The equilibrium synthesis yields were determined after a 48 h reaction on the basis of the limited starting substrate consumption. The equilibrium constants were then calculated according to Eq. (1).

$$K_{eq} = \frac{a_w \times a_p}{a_{\text{myristic acid}} \times a_B} \quad (1)$$

where a_w and $a_{\text{myristic acid}}$ are the thermodynamic activities of water and myristic acid, respectively. a_p and a_B are the thermodynamic activities of 1-methylpropyl myristate ester or myristic acid methylpropylamide, and of 2-butanol or *sec*-butylamine, respectively. The thermodynamic activity values used were estimated using the contribution method of predicting activity coefficients, UNIFAC (universal functional activity coefficient) [13].

The initial velocities were calculated from the linear relationship of the total concentration of products against reaction time (0–2 h for the 2-butanol esterification and 0–3 h for the *sec*-butylamine amidification).

2.3. Data analysis

2.3.1. Determination of kinetic mechanisms

All initial rate data were fitted to all the kinetic models provided by the Enzyme Kinetics Module of Sigma Plot, “Enzyme Kinetics 2004 1.3” (Systat Software Inc., San Jose, USA), using nonlinear least-squares regression analysis.

Initial rate data corresponding to 2-butanol O-acylation were best fitted to the equation describing a steady-state ordered ternary complex bi–bi mechanism [14] (Eq. (2)).

$$v = \frac{V_{\max}[A][B]}{K_{ia}K_{mB} + K_{mB}[A] + K_{mA}[B] + [A][B]} \quad (2)$$

Initial rate data corresponding to *sec*-butylamine N-acylation for low concentrations of amine (<100 mM) and myristic acid (<175 mM) were best fitted to the equation describing a steady-state ping–pong bi–bi mechanism [14] (Eq. (3)).

$$v = \frac{V_{\max}[A][B]}{K_{mB}[A] + K_{mA}[B] + [A][B]} \quad (3)$$

A represents myristic acid as B represents 2-butanol in Eq. (2) and *sec*-butylamine in Eq. (3). K_{mA} and K_{mB} are the Michaelis constants for A and B, respectively. V_{\max} is the maximum rate. K_{ia} is defined as the dissociation constant of A from the binary complex EA.

To allow visualization of fitting quality, the experimental data points are presented in plots along with theoretical lines fitted by “Enzyme Kinetics 2004 1.3” software. Moreover, linear regressions of these theoretical lines were used to calculate the apparent kinetic parameters V_{\max}^{app} and K_{mB}^{app} .

2.3.2. Evaluation of the enantioselectivity and of the chemoselectivity

The enantioselectivity can be described by the enantiomeric ratio (E). If the initial rates of the individual enantiomers acylations are measured separately at a certain substrate concentration, the ratio of initial rates depends on the substrate concentration and serves merely as a qualitative measure of enantioselectivity (Eq. (4)). If concentrations well below the K_m values are used (pseudo first-order kinetics) or if $K_m^R = K_m^S$, the ratio of initial rates will equal E and the catalytic efficiency ratio [15]. In this study, E -values were always calculated by using the catalytic efficiency ratio.

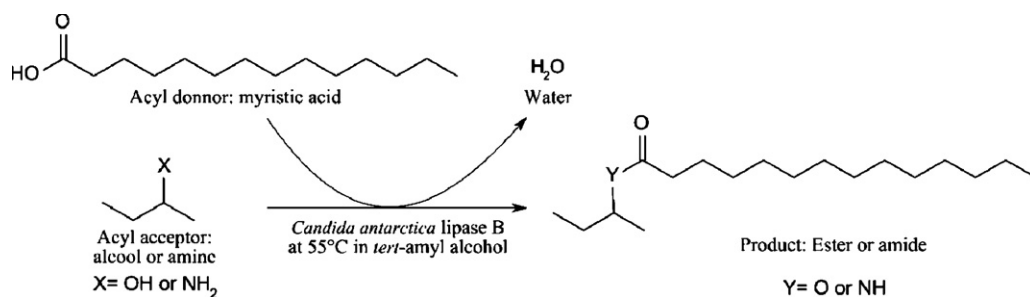
$$E = \frac{v_i^R}{v_i^S} = \frac{V_{\max}^R/(K_m^R + C_{S0})}{V_{\max}^S/(K_m^S + C_{S0})} \quad (4)$$

The chemoselectivity of *C. antarctica* lipase B was studied by comparing (R)-2-butanol O-acylation and (R)-*sec*-butylamine N-acylation, and then evaluated via the apparent catalytic efficiency ratio (Eq. (5)) [16].

$$C = \left(\frac{V_{\max}^{\text{app O-acylation}}}{K_m^{\text{app O-acylation}}} \right) \left(\frac{V_{\max}^{\text{app N-acylation}}}{K_m^{\text{app N-acylation}}} \right) \quad (5)$$

2.4. HPLC analysis

Structural and quantitative analysis of reaction products were conducted using a LC/MS–ES system from Agilent (1100 LC/MSD Trap mass spectrometer VL) with a C18 Uptisphere 300 A OD column (250 mm × 4 mm, 5 µm; Interchim) for the analysis of esters and a C18 ProntoSIL 120–5–C18–AQ column (250 mm × 4 mm, 5 µm; Bischoff Chromatography) for the analysis of amides. Both columns were eluted with acetonitrile/water/acetic acid (90:10:0.1, v/v/v) at room temperature and at a flow rate of 1 ml min⁻¹. Products were detected and quantified by differential refractometry using HP Chemstation software off-line for the processing.



Scheme 1. Acylation of monofunctional alcohols and amines catalyzed by *Candida antarctica* lipase B in *tert*-amyl alcohol.

3. Results and discussion

3.1. Kinetics and enantioselectivity of 2-butanol O-acylation

The synthesis of 1-methylpropyl myristate ester catalyzed by *C. antarctica* lipase B from 2-butanol with myristic acid as an acyl donor was chosen as the model reaction for the O-acylation study. Pure (R)- and (S)-enantiomers of 2-butanol were used in separate experiments to investigate the effect of the concentration of both substrates on the initial rate values and to elucidate the kinetic mechanism of the reaction. From the HPLC analysis, the decrease of myristic acid concentration was always seen to be concomitant with the synthesis of 1-methylpropyl myristate ester which was identified by mass spectroscopy. In absence of enzyme, no product was detected within 2 days.

Both substrate concentrations were varied from 25 mM to 350 mM and Fig. 1 shows the effect of the substrate molar ratio on the equilibrium ester synthesis yield based on the limiting initial substrate concentration, after 48 h of (R)- or (S)-2-butanol acylation. We observed that the yield increased sharply when one substrate was used in excess, starting from about 15% ester synthesis under stoichiometric conditions to reach more than 60% ester synthesis under the conditions with 350 mM of myristic acid or 2-butanol. Under the best conditions used, corresponding to 350 mM of 2-butanol and 25 mM of myristic acid, up to 69% conversion (17.2 mM) was obtained with the (S)-conformation, giving an equilibrium constant K_{eq} of 1.87, which favors synthesis. Moreover, no significant difference was observed between the (R)- and (S)-ester synthesis yields whatever the substrate molar ratio.

Systematic analysis of the rates, which were dependent on the substrate concentration, revealed two Lineweaver–Burk double

reciprocal plots, depending on whether myristic acid or (R)-2-butanol was parametric. The results are shown in Fig. 2.

The corresponding families of the double reciprocal plots were linear and gave intersecting patterns at the left of the y-axis (Fig. 2A and B), characterizing either a steady-state ordered ternary complex bi–bi mechanism or a Theorell–Chance mechanism. The probability that a Theorell–Chance system exists is very low and this mechanism was therefore excluded [14]. We did not observe any type of inhibition with the tested substrate concentrations. The initial rate data obtained with the (S)-enantiomer were also seen to fit with this kinetic model (data not shown).

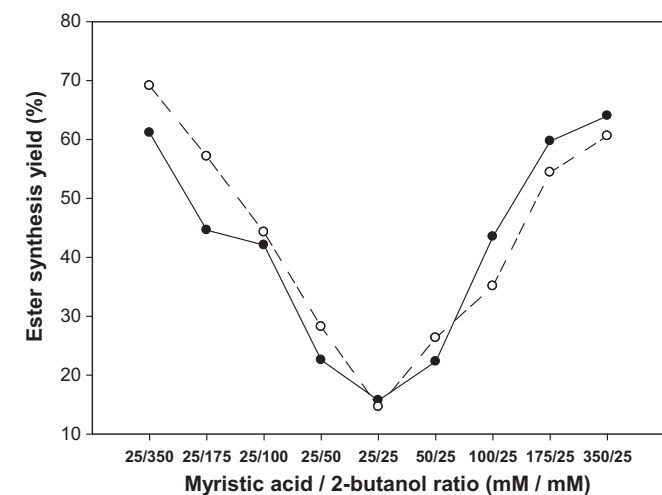


Fig. 1. Effect of the myristic acid/2-butanol molar ratio on the equilibrium ester synthesis yield. Reactions were carried out in *tert*-amyl alcohol using (R)-2-butanol (●) or (S)-2-butanol (○) for 48 h at 55 °C with 10 g l^{-1} of *Candida antarctica* lipase B.

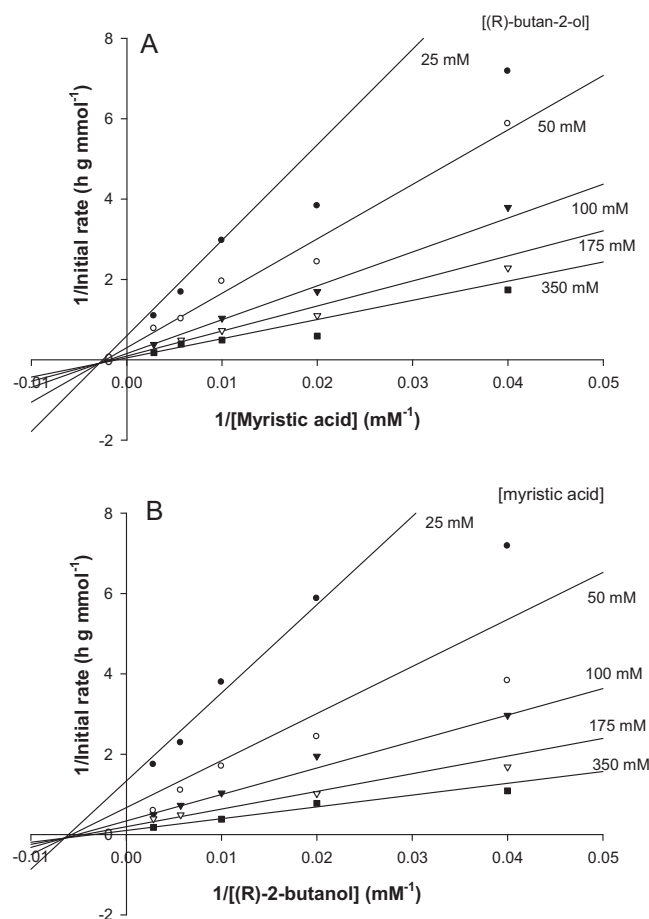


Fig. 2. Reciprocal initial rates versus reciprocal substrate concentrations. The synthesis was carried out at 55 °C using 10 g l^{-1} of *Candida antarctica* lipase B. (A) The concentration of (R)-2-butanol was fixed at 25 mM (●), 50 mM (○), 100 mM (▼), 175 mM (▽) and 350 mM (■). (B) The concentration of myristic acid was fixed at 25 mM (●), 50 mM (○), 100 mM (▼), 175 mM (▽) and 350 mM (■). The data represent the averages of triplicate runs whose standard deviations were always lower than 15%.

In the literature, most of the models concerning the lipase-catalyzed acylation of primary alcohols are based on a ping-pong bi-bi mechanism [4,5]. This model was notably used to describe O-acylation reactions catalyzed by *C. antarctica* lipase B in organic solvents [8], in supercritical media [17] and in solid-gas reactors [18]. However, the ordered bi-bi mechanism was reported in few studies dealing with transesterification reactions catalyzed by *C. antarctica* lipase B and carried out in solvent-free systems [6,19]. In fact, the catalytic mechanism of lipases is believed to be analogous to that proposed for serine proteases, involving the known active site triad of residues serine, histidine and aspartic acid. It has been suggested that a Michaelis–Menten complex formed between the acyl donor and the enzyme proceeds to give the first tetrahedral intermediate. This then collapses to give an acyl-enzyme intermediate and release the first product. Then the second substrate binds to the active site, allowing the formation of the second tetrahedral intermediate. Finally, the latter breaks down to give the ester product [20]. This corresponds to a ping-pong bi-bi mechanism but in our situation, the first product is water, which is a small molecule that may already be present in the catalytic site and cannot be a cause of steric hindrance. So the second substrate can access to the active site before the release of the first product, leading to an ordered bi-bi mechanism.

The apparent kinetic parameters V_{\max}^{app} and $K_{\text{mB}}^{\text{app}}$ were determined (Table 1).

These results provide informations about the enantioselectivity of *C. antarctica* lipase B toward the O-acylation. First, the apparent maximum rate (V_{\max}^{app}) of the ester production starting from the (R)-conformation ($4.62 \text{ mmol h}^{-1} \text{ g}^{-1}$) was about 2-fold higher than the apparent maximum rate obtained starting from the (S)-conformation ($2.5 \text{ mmol h}^{-1} \text{ g}^{-1}$). Furthermore, the apparent Michaelis constant ($K_{\text{mB}}^{\text{app}}$) of the (R)-enantiomer (150 mM) was slightly lower than the $K_{\text{mB}}^{\text{app}}$ of the (S)-enantiomer (207 mM), which indicated an affinity of the lipase slightly favoring the (R)-enantiomer. Logically, the resulting catalytic efficiency for the (R)-enantiomer acylation ($0.031 \text{ l h}^{-1} \text{ g}^{-1}$) was higher than the catalytic efficiency for the (S)-enantiomer acylation ($0.012 \text{ l h}^{-1} \text{ g}^{-1}$). Moreover, an *E*-value of about 3.17 (Eq. (4)) was obtained when using 175 mM of myristic acid, which signified that *C. antarctica* lipase B had a preferential enantioselectivity toward the (R)-enantiomer O-acylation.

This result is consistent with several works, which have shown that *C. antarctica* lipase B preferentially catalyzes the (R)-enantiomer acylation [21]. Some molecular modeling data have revealed that this enantioselectivity may be related to the enzyme conformation which would allow the (R)-enantiomer to easier access the active site [22].

3.2. Kinetics and enantioselectivity of *sec*-butylamine N-acylation

The synthesis of myristic acid methylpropylamide catalyzed by *C. antarctica* lipase B from *sec*-butylamine with myristic acid as an acyl donor was chosen as the model reaction for the N-acylation study. Pure (R)- and (S)-enantiomers of *sec*-butylamine were used in separate experiments to investigate the effect of both substrate concentrations on the initial rate and the synthesis yield. From

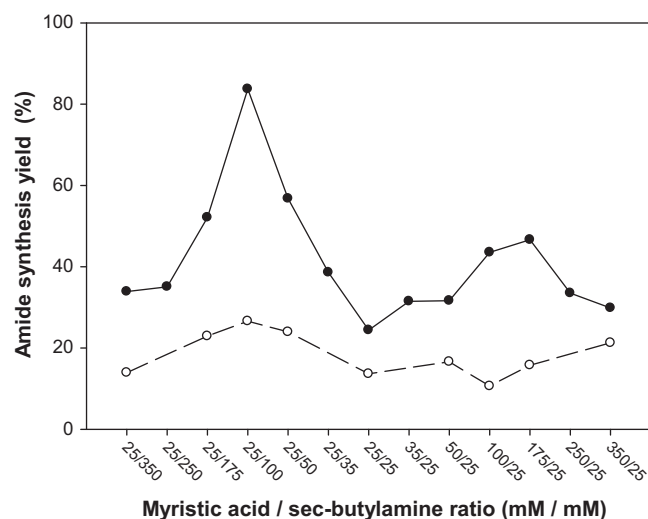


Fig. 3. Effect of the myristic acid/*sec*-butylamine molar ratio on the equilibrium amide synthesis yield. Reactions were carried out in *tert*-amyl alcohol using (R)-*sec*-butylamine (●) or (S)-*sec*-butylamine (○) for 48 h at 55 °C with 50 g l^{-1} of *Candida antarctica* lipase B.

the HPLC analyses, the decrease in myristic acid concentration was always seen to be concomitant with the synthesis of myristic acid methylpropylamide, which was identified by mass spectroscopy. In absence of enzyme, no product was detected within 2 days.

Fig. 3 shows the effect of the myristic acid/*sec*-butylamine molar ratio on the equilibrium amide synthesis yield based on the limiting initial substrate concentration. Under the best conditions used (100 mM of (R)-*sec*-butylamine and 25 mM of myristic acid), 84% of amide synthesis (21 mM) was reached for the (R)-enantiomer acylation, giving an equilibrium constant K_{eq} of 14.37, strongly favoring synthesis, while the amide synthesis yield never exceeded 25% (6.2 mM) for the (S)-enantiomer acylation, whatever the myristic acid/*sec*-butylamine molar ratio.

Furthermore, the (R)-amide synthesis yields were higher than the (S)-amide synthesis yields under all conditions tested. We also observed that the increase in one substrate concentration, from 25 to 100 mM of *sec*-butylamine and from 25 to 175 mM of myristic acid, allowed to enhance the amide synthesis yield. However, the use of a large excess of one substrate toward the other, corresponding to concentrations higher than 100 mM of *sec*-butylamine or 175 mM of myristic acid, caused the decrease in the amide synthesis yield. This was most likely due to an inhibitor effect similar to an excess substrate inhibition that was not observed for the O-acylation of 2-butanol (see Section 3.1; Fig. 1), probably due to an interaction between *sec*-butylamine and myristic acid. Indeed, the presence of an amino substrate and a fatty acid in an organic solvent generally leads to the formation of an ion-pair complex between both substrates, depending on the acido-basic conditions of the medium [12,19,23]. This salt complex can lead to the unreactivity of the ion forms of both substrates (NH_3^+ amine form and COO^- fatty acid form) and therefore to the overestimation of the reactive substrate concentrations that are really available for the

Table 1

Apparent kinetic parameters of acylations using 175 mM myristic acid as acyl donor and 25–350 mM substrate B as acyl acceptor in *tert*-amyl alcohol.

Reaction	Substrate B	$K_{\text{mB}}^{\text{app}}$ (mM)	V_{\max}^{app} ($\text{mmol h}^{-1} \text{ g}^{-1}$)	Apparent catalytic efficiency ($V_{\max}^{\text{app}}/K_{\text{mB}}^{\text{app}}$) ($\text{l h}^{-1} \text{ g}^{-1}$)
1	(R)-2-butanol	150	4.62	0.031
2	(S)-2-butanol	207	2.5	0.012
3	(R)- <i>sec</i> -butylamine	619	0.21	3.36×10^{-4}
4	(S)- <i>sec</i> -butylamine	120	0.03	2.5×10^{-4}

enzyme in the reaction medium. This ion-pair complex was already described by Maugard et al. [12] as a limiting factor of the lipase-catalyzed acylation under conditions where it was less soluble than free substrates.

Using kinetic experiments, we then sought to identify the kinetic mechanism of the synthesis of myristic acid methylpropylamide from *sec*-butylamine and myristic acid catalyzed by *C. antarctica* lipase B. Systematic analysis of the rates, which were dependent on the substrate concentration, revealed two Lineweaver–Burk double reciprocal plots, depending on whether myristic acid or (R)-*sec*-butylamine was parametric (Fig. 4). The corresponding families of the double reciprocal plots showed a set of solid parallel lines for low concentrations of substrates (inferior or equal to 100 mM of myristic acid or 50 mM of (R)-*sec*-butylamine) characterizing a steady-state ping–pong bi–bi mechanism. The initial rate data obtained with the (S)-enantiomer were also seen to fit with this kinetic model (data not shown).

Moreover, for high concentrations of substrates (superior to 100 mM of myristic acid or (R)-*sec*-butylamine), a decrease in initial rates was observed: this was characterized on both Lineweaver–Burk plots by an increase in 1/initial rate-values as well as a loss of parallelism of the corresponding plots, which showed a set of dashed lines tending to intersect near the y-axis.

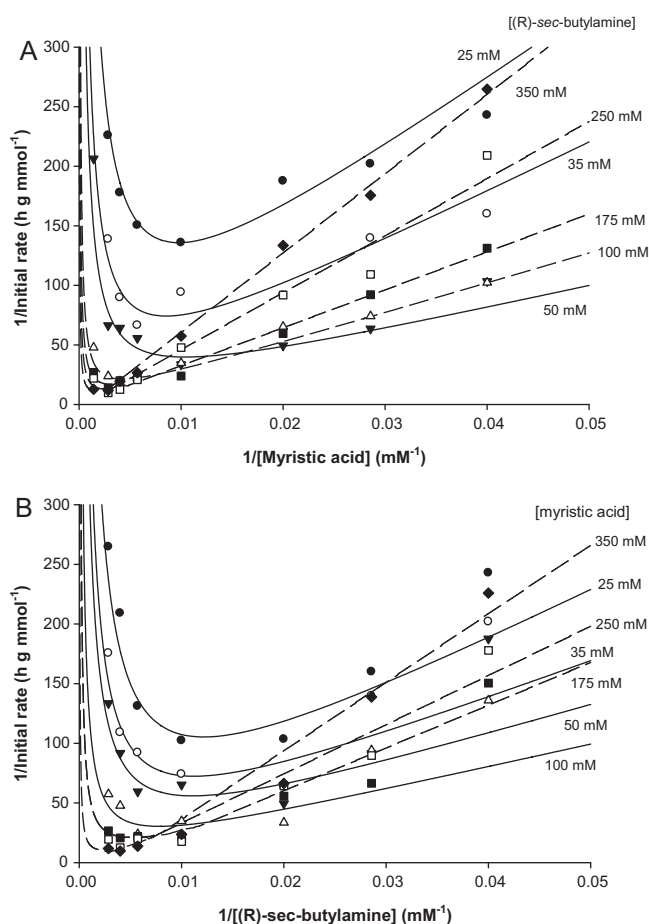


Fig. 4. Reciprocal initial rates versus reciprocal substrate concentrations. The synthesis was carried out at 55 °C using 50 g l⁻¹ of *Candida antarctica* lipase B. (A) Myristic acid concentration was varied from 25 to 350 mM and (R)-*sec*-butylamine concentration was fixed at 25 mM (●), 35 mM (○), 50 mM (▼), 100 mM (△), 175 mM (■), 250 mM (□) and 350 mM (◆). (B) (R)-*sec*-butylamine concentration was varied from 25 to 350 mM and myristic acid concentration was fixed at 25 mM (●), 35 mM (○), 50 mM (▼), 100 mM (△), 175 mM (■), 250 mM (□) and 350 mM (◆). The data represent the averages of triplicate runs whose standard deviations were always lower than 15%.

Two hypotheses can be proposed to explain this phenomenon. First, it may be due to a steady-state ping–pong bi–bi mechanism implying a double substrate competitive inhibition, which would be enforced by the fact that the corresponding experimental data were best fitted with this model. Nevertheless, this hypothesis should probably be ruled out given that no inhibition by myristic acid was observed for 2-butanol O-acylation conducted under very similar conditions. The second and most likely hypothesis would be the previously proposed formation of an ion–pair complex between (R)-*sec*-butylamine and myristic acid that would lead to the decrease in the substrates availability in the enzyme environment. As the formation of a complex salt cannot occur between myristic acid and 2-butanol, due to the impossibility for 2-butanol to form its unprotonated anionic form under the experimental conditions used, this phenomenon was not observed for 2-butanol O-acylation.

The apparent kinetic parameters V_{\max}^{app} and $K_{\text{mB}}^{\text{app}}$ were determined (Table 1). These results provide informations about the enantioselectivity of *C. antarctica* lipase B toward the N-acylation. First, V_{\max}^{app} of the amide production starting from the (R)-conformation (0.21 mmol h⁻¹ g⁻¹) was about 7-fold higher than the apparent maximum rate obtained starting from the S conformation (0.03 mmol h⁻¹ g⁻¹). On the other hand, the $K_{\text{mB}}^{\text{app}}$ of the (R)-enantiomer (619 mM) was about 5-fold higher than the $K_{\text{mB}}^{\text{app}}$ of the (S)-enantiomer (120 mM), which indicated an affinity of the lipase significantly favoring the (S)-enantiomer. Nevertheless, the resulting catalytic efficiency for the (R)-enantiomer acylation (3.36×10^{-4} h⁻¹ g⁻¹) was higher than the catalytic efficiency for the (S)-enantiomer acylation (2.5×10^{-4} h⁻¹ g⁻¹), which signified that *C. antarctica* lipase B had a preferential enantioselectivity toward (R)-enantiomer N-acylation, mostly due to a better catalysis rate toward the (R)-enantiomer. This was confirmed by calculating an *E*-value of 1.34 (Eq. (4)) when using 175 mM of myristic acid.

3.3. Chemoselectivity of *C. antarctica* lipase B

The kinetic data of 2-butanol O-acylation and *sec*-butylamine N-acylation showed in the first and second parts of our study that *C. antarctica* lipase B favored the O-acylation. The V_{\max}^{app} of (R)-ester synthesis (4.62 mmol h⁻¹ g⁻¹) was thus about 22-fold higher than the V_{\max}^{app} of (R)-amide synthesis (0.21 mmol h⁻¹ g⁻¹). On the other hand, the $K_{\text{mB}}^{\text{app}}$ of (R)-*sec*-butylamine (619 mM) was 4-fold higher than the $K_{\text{mB}}^{\text{app}}$ of (R)-2-butanol (150 mM). This pointed out a better affinity of *C. antarctica* lipase B toward 2-butanol (Table 1, reaction 1). To more precisely evaluate the chemoselectivity of *C. antarctica* lipase B, (R)-2-butanol O-acylation and (R)-*sec*-butylamine N-acylation were conducted separately using 175 mM of myristic acid. The chemoselectivity ratio (*C*) was then calculated (Eq. (5)), giving a *C*-value close to 92. This value clearly confirmed that *C. antarctica* lipase B was chemoselective for the O-acylation under our synthesis conditions. In vivo, lipases are acyl-hydrolases that catalyze the hydrolysis of esters [24,25]. It seems thus coherent for *C. antarctica* lipase B to be chemoselective for the O-acylation of (R)-2-butanol rather than for the N-acylation of (R)-*sec*-butylamine. In addition, some studies dealing with the acylation of long difunctional compounds have shown similar results. In particular, Husson et al. reported a high chemoselectivity in rates exhibited by *C. antarctica* lipase B for the O-acylation of 6-amino-1-hexanol conducted in *tert*-amyl alcohol starting from oleic acid as an acyl donor [19]. Furthermore, apart from an intrinsic chemoselectivity naturally due to the design and the in vivo function of *C. antarctica* lipase B, the previously discussed possibility of a negative effect induced by the formation of an ion–pair complex between (R)-*sec*-butylamine and myristic acid may also explain the low chemoselectivity observed toward N-acylation in comparison with O-acylation.

4. Conclusion

The kinetic behavior of *C. antarctica* lipase B toward 2-butanol O-acylation and *sec*-butylamine N-acylation starting from myristic acid as an acyl donor was investigated. The equilibrium yields and initial rates of the reactions were measured in order to identify the kinetic mechanisms and parameters which allowed us to understand the origin of the lipase selectivity. Under the best O-acylation conditions used, a similar synthesis yield (close to 65%; 16 mM) was observed after 48 h for the conversion of (R)- or (S)-2-butanol into 1-methylpropyl myristate ester. On the other hand, optimal N-acylation conditions resulted in a significant preference for the conversion of (R)-*sec*-butylamine into myristic acid methylpropylamide, which reached about 84% (21 mM) when excess (R)-*sec*-butylamine was used, whereas only 25% of (S)-*sec*-butylamine was converted under optimal conditions. These results are of interest as they highlight the potential use of *C. antarctica* lipase B for the biotechnological selective acylation of amino-alcohols with high yields, which could have applications in many areas, such as pharmaceuticals and cosmetics (the synthesis of ceramides [26] as potential anti-viral or anti-tumor drugs [27,28] or anti-oxidant stabilizers [29]), or for the environment, food, and agricultural industries (the synthesis of glucamide- or aminoacid-based surfactants [2,30]).

Regarding the kinetic studies of the reactions, the experimental data corresponding to the N-acylation of (R)-*sec*-butylamine and (S)-*sec*-butylamine were fitted with a steady-state ping-pong bi-bi mechanism for lower substrate concentrations. Interestingly, the kinetic data corresponding to the O-acylation of (R)-2-butanol and (S)-2-butanol were found to fit with a steady-state ordered ternary complex bi-bi mechanism model. Concerning the enantioselectivity studies, *C. antarctica* lipase B was found to preferentially catalyze (R)-2-butanol O-acylation in terms of both catalytic activity and affinity: the calculated *E*-value of 3.17 confirmed its preferential enantioselectivity for (R)-enantiomer O-acylation. *sec*-Butylamine N-acylation also showed a preferential enantioselectivity of *C. antarctica* lipase B for the (R)-enantiomer, giving an *E*-value of 1.34. Nevertheless, (R)-*sec*-butylamine N-acylation was shown to occur with higher initial rates and yields than those of (S)-*sec*-butylamine N-acylation whereas the affinity of *C. antarctica* lipase B was lower for (R)-*sec*-butylamine than for (S)-*sec*-butylamine. The preferential enantioselectivity of *C. antarctica* lipase B for (R)-*sec*-butylamine N-acylation seemed in fact to find its origin mostly in the difference of catalysis rate and thus of catalytic activity rather than in the difference of affinity toward the (R)- and (S)-enantiomers. Finally, it was confirmed that *C. antarctica* lipase B is a chemoselective enzyme [2], exhibiting a preference for O-acylation rather than for N-acylation [19].

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