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How the mode of *Candida antarctica* lipase B immobilization affects the continuous-flow kinetic resolution of racemic amines at various temperatures

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ABSTRACT

The effect of temperature on enantiomeric ratio (E) and specific reaction rate (r_{flow}) in the continuous-flow mode acetylation of (±)-1-phenylethanamine (*rac*-**1a**), (±)-4-phenylbutan-2-amine (*rac*-**1b**) and (±)-1,2,3,4-tetrahydro-1-naphthalenamine (*rac*-**1c**) by variously immobilized *Candida antarctica* lipase B biocatalysts was studied in the 0–70 °C range. In the continuous-flow kinetic resolutions with differently immobilized CaLB biocatalysts, the character of temperature effect depended significantly both on the substrate and on the mode of immobilization. Alteration of E in the kinetic resolutions of three differently flexible amines *rac*-**1a–c** as a function of temperature was rationalized by the various flexibility of the lipase in its different forms. Our results indicated that the optimal method of immobilization depended both on the nature of the substrate and the reaction conditions.

1. Introduction

Enantiomerically pure chiral amines are highly valuable building blocks of numerous drugs [1,2] and agrochemicals [3]. They are also useful as resolving agents for enantiomer separation via diastereomeric salt formation, and as ligands for asymmetric synthesis using either transition metal catalysis or organocatalysis [2]. The majority of drugs are amines or their derivatives, and if chiral, their application in a non-racemic form becomes an ever increasing requirement [2].

Production of optically active intermediates is an ever expanding area of the pharmaceutical and fine chemical industry. As an important tool for enantioselective syntheses, biocatalysis has become a widely used technology [4–6]. Recently attention turned to the kinetic resolution of racemic amines [7–12]. Hydrolases have long been used for the kinetic resolution of racemic alcohols and carboxylic acids. In the past decades, lipase B from *Candida antarctica* (CaLB) has become a widely used biocatalyst in pharmaceutical and fine chemical industries [13,14]. Most of the reported examples for kinetic resolution of racemic amines were run in organic solvents, with CaLB as the most commonly employed hydrolase [2,13,15].

Effect of the temperature on the enantioselectivity in enzymatic reactions is well known. In a wide range of enzymatic reactions decreasing selectivity with increasing temperature was observed [16–25] and there are only a few examples in which higher temperature resulted in higher selectivity [26,27]. Occasionally, temperature had no effect on enantioselectivity [28], whereas in other cases a maximum of enantioselectivity at a certain temperature was found [29]. Effect of the temperature on selectivity in CaLB-catalyzed hydrolysis [30–33], ammonolysis [34], direct esterification [35,36], enantioselective acylation of prochiral diols [37] and kinetic resolution of alcohols [29,38,39] has already been reported. In most of the quoted examples the enzyme-catalyzed reactions were performed in batch mode (in stirred or shaken flasks). Only one study on the temperature effects on kinetic resolution, namely that of secondary alcohols by CaLB (Novozym® 435) was performed in a continuous-flow mode [29].

To the best of our knowledge only a single study dealt with the temperature dependence of enantiomer selectivity in enzyme-catalyzed *N*-acylations: decreasing selectivity with increasing temperature was found in the *N*-acylation of ethyl 3-amino-3-phenylpropanoate catalyzed by immobilized lipase A from *C. antarctica* (CaLA) [40]. However, the temperature dependence of

Abbreviations: CaLB, lipase B from *Candida antarctica*; DMDEOS, dimethyldiethoxysilane; PhTEOS, phenyltriethoxysilane; TEOS, tetraethoxysilane.

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CaLB-catalyzed by *N*-acylation of racemic amines seems to have been not studied so far.

It is known that the state of the enzyme – e.g. the mode of immobilization – may significantly influence the properties of the biocatalysts [41–43]. E.g. a lipase entrapped in a phenyl-containing sol–gel matrix was preferable for kinetic resolution of 1-phenylethanol, whereas another form of the same lipase entrapped in an octyl-containing matrix was more efficient with more aliphatic substrates [44]. Another example indicated that the biocatalytic properties of a lipase in a given sol–gel matrix can be modulated by bioimprinting effects of substrate-mimicking molecules [45]. Dependence of the enantioselectivity of lipase biocatalysts immobilized by physical adsorption on the nature of support was also studied [46,47]. It has been reported that immobilization protocol can strongly alter the enantiospecificity of CaLB in transesterifications [48] or in hydrolysis in aqueous media [49]. Surprisingly, there are only a few examples on comparative temperature effect studies with the same enzyme in different forms. For example, the enantioselectivities of various preparations of subtilisin Carlsberg in transesterification of racemic 1-phenylethanol with vinyl butyrate were studied in dioxane at different temperatures from 7 to 45 °C [50]. It was found that enantioselectivity and its temperature dependence varied greatly with the mode of immobilization. Although this example indicated the importance of the immobilization mode on the catalytic properties of an enzyme, there is no study on the significance of the immobilization method of a lipase in kinetic resolution of amines has been reported so far.

In the present study we investigated the temperature-dependence of the selectivity in the kinetic resolution of racemic amines performed in continuous-flow bioreactors using variously immobilized forms of lipase B from *C. antarctica* (CaLB). In order to be able to compare various methods of immobilization on lipase-catalyzed amine resolution, productivities (r), enantiomer selectivities (E) and enantiomeric purities (ee_P) of the products in CaLB-catalyzed *N*-acetylation in batch and continuous-flow modes were measured.

2. Materials and methods

2.1. Materials

(±)-1-Phenylethanamine (*rac*-**1a**), (±)-4-phenylbutan-2-amine (*rac*-**1b**) and (±)-1,2,3,4-tetrahydro-1-naphthalenamine (*rac*-**1c**) were purchased from Sigma–Aldrich (Saint Louis, MO, USA). Dimethyldiethoxysilane (DMDEOS), phenyltriethoxysilane (PhTEOS) and tetraethoxysilane (TEOS) were the products of Alfa Aesar Europe (Karlsruhe, Germany).

CaLB G250P (lipase B from *C. antarctica*, adsorbed on phenyl-functionalized silica gel) and CaLB SGA10D (lipase B from *C. antarctica* immobilized in sol–gel matrix, using TEOS:PhTEOS:DMDEOS as silane precursors [45]) were the products of SynBiocat Ltd. (Budapest, Hungary). CaLB T2-150 (lipase B from *C. antarctica*, covalently attached to dry acrylic beads of 150–300 μm particle size) was the product of ChiralVision BV (Leiden, The Netherlands). Novozym® 435 (CaLB N435, lipase B from *C. antarctica*, recombinant, expressed in *Aspergillus niger*, adsorbed on acrylic resin) was obtained from Sigma–Aldrich (Saint Louis, MO, USA).

Solvents (toluene, ethyl acetate) from Molar Chemicals (Budapest, Hungary) were dried and/or freshly distilled prior to use.

2.2. Immobilized CaLB-filled packed-bed columns

Enzymes (CaLB N435, CaLB T2-150, CaLB G250P, CaLB SGA10D) were packed into stainless steel CatCart™ columns according to the filling process of ThalesNano Inc. Before packing, the columns were washed with distilled water, ethanol, *n*-hexane and acetone in an ultrasonic cleaner. For the continuous flow enzymatic applications, the columns were sealed by silver metal filter membranes [Sterlitech Silver Membrane Filter from Sigma–Aldrich, Z623237, pore size 0.45 μm; pure metallic silver, 99.97% with no extractable or detectable contaminants] due to the known benefits of Ag (bacteriostatic). The sealings were made of PTFE. Three CatCart™ columns per enzyme (separate columns for each substrate, *rac*-**1a–c**) were packed for this study (filling weights: CaLB N435, 288 ± 2 mg; CaLB T2-150, 257 ± 6 mg; CaLB G250P, 232 ± 6 mg and CaLB SGA10D, 213 ± 13 mg).

2.3. Methods

TLC was carried out using Kieselgel 60 F_{254} (Merck) sheets. Spots were visualized under UV light (Vilber Lourmat VL-6.LC, 254 nm and 365 nm) or by treatment with 5% ethanolic phosphomolybdic acid solution and heating of the dried plates.

Reactions were analyzed by GC on an Agilent 5890 equipment using Hydrodex β-TBDAc column (Machery–Nagel; 25 m × 0.25 mm × 0.25 μm, heptakis-(2,3-di-O-acetyl-6-O-*t*-butyl-dimethylsilyl)-β-cyclodextrin) [FID (250 °C), injector (250 °C), H2 (12 psi, split ratio: 1:50)]. GC data (oven program), t_r (min): for **1a** and **2a** (100–180 °C, 8 °C min^{-1}, 5 min at 180 °C), 2.9 [(*S*)-**1a**], 3.1 [(*R*)-**1a**], 9.8 [(*R*)-**2a**], 10.0 [(*S*)-**2a**]; for **1b** and **2b** (140–170 °C, 1 °C min^{-1}), 2.6 [**1b**], 23.4 [(*S*)-**2b**], 23.6 [(*R*)-**2b**]; for **1c** and **2c** (120 °C, 10 min, 120–180 °C, 6 °C min^{-1}, 180 °C, 10 min), 7.6 [(*R*)-**1c**], 7.8 [(*S*)-**1c**], 23.6 [(*R*)-**2c**], 24.1 [(*S*)-**2c**]. Only those data were indicated in Figs. 2–4 which arose from a precise integration of chromatograms in which both enantiomers of the products **2a–c** were clearly visible. When the minor enantiomers (*S*)-**2a–c** were indistinguishable from the noise, solid curves were replaced by dashed lines.

Conversion (c), enantiomeric excess (ee) and enantiomeric ratio (E) were determined by GC. Enantiomeric ratio (E) was calculated from c and enantiomeric excess of the product (ee_P) using the equation $E = \ln[1 - c(1+ee_P)]/\ln[1 - c(1 - ee_P)]$ [51]. To characterize the productivity of the biocatalysts, the specific reaction rates in batch reactions (r_{batch}) were calculated using the equation $r_{batch} = n_P/(t \times m_B)$ (where n_P [μmol] is the amount of the product, t [min] is the reaction time and m_B [g] is the mass of the applied biocatalyst) [29]. The specific reaction rates in continuous-flow systems (r_{flow}) were calculated using the equation $r_{flow} = [P] \times v/m_B$ (where $[P]$ [μmol mL^{-1}] is the molar concentration of the product, v [mL min^{-1}] is the flow rate and m_B [g] is the mass of the applied biocatalyst) [29]. As stated earlier [29], as the rate of product formation is not a linear function of c, rigorous comparisons between the productivity of a continuous-flow reaction and its batch mode counterpart using their r values can only be made at similar degrees of conversions.

2.4. Enantiomer selective acetylation of racemic amines rac-*1a–c* in batch mode

To a solution of the racemic amine *rac*-**1a–c** (20 mg) in anhydrous toluene:ethyl acetate 9:1 mixture (2 mL) in an amber screw cap glass vial the enzyme (20 mg) was added, the vial closed and the mixture was shaken (1000 rpm) at 30 °C for 24 h. Samples were taken directly from the reaction mixture (sample size: 10 μl, diluted with CH_2Cl_2 to 250 μl) after 0.5, 1, 2, 4, 6, 8 and 24 h and analyzed by GC as described in Section 2.3.

2.5. Enantiomer selective acetylation of racemic amines rac-**1a–c** in continuous-flow bioreactor

The continuous-flow kinetic resolutions were performed in a laboratory flow reactor comprising an isocratic HPLC pump (Knauer K-120) attached to CatCart™ columns filled with the immobilized CaLB biocatalysts in an in-house made thermostated aluminum metal block column holder with precise temperature control. Before use, the CaLB-filled columns were washed with an anhydrous 9:1 mixture of toluene and ethyl acetate (0.5 mL min^{-1}, 20 min).

The solution of the racemic amine (10 mg mL^{-1} of *rac*-**1a–c** i.e. 0.083 M for *rac*-**1a**, 0.067 M for *rac*-**1b**, 0.068 M for *rac*-**1c**) in an anhydrous 9:1 mixture of toluene and ethyl acetate was pumped through the enzyme filled column thermostated to various temperatures (0–70 °C) at a flow rate of 0.2 mL min^{-1}. At each temperature, samples were analyzed by GC every 10 min up to 40 min after the beginning of the experiment. Samples were collected after stationary operation has been established (sample size: 10 μl, diluted with CH_2Cl_2 to 250 μl; taken generally 30 min after changing the parameters) and analyzed as described in Section 2.3.

Experiments were run in 10 °C steps at temperatures of 0–70 °C. Then the column was washed with toluene (0.5 mL min^{-1}, 20 min) and stored in a refrigerator (4 °C).

3. Results and discussion

In this study our aim was to investigate the effect of the various immobilization methods on a lipase-catalyzed kinetic amine resolution. As a model biocatalyst the lipase B from *C. antarctica* (CaLB) was selected which is capable of catalyzing *N*-acetylations of a broad range of amines both in batch and continuous-flow mode [2,13].

For this purpose the performance of four differently immobilized forms of CaLB was compared. The most widely used form of CaLB is Novozym® 435 (CaLB N435), containing the lipase adsorbed on an acrylic resin. For comparison CaLB G250P i.e. lipase adsorbed on phenyl-functionalized silica gel, CaLB T2-150 i.e. lipase covalently attached to acrylic beads as well as CaLB SGA10D i.e. lipase entrapped in a hydrophobic sol–gel matrix were selected. In this way, diverse selection of various immobilization methods (adsorption on organic support, adsorption on inorganic support, covalent attachment on polymeric support and sol–gel entrapment) was covered.

The variously immobilized forms of CaLB were tested in kinetic resolution by acylation with ethyl acetate of some typical racemic secondary amines i.e. *rac*-**1a–c**, in dry toluene (Fig. 1). (±)-1-Phenylethanamine (*rac*-**1a**) containing a rigid aromatic ring and a methyl group adjacent to the asymmetric carbon center is one of the most thoroughly studied amine in enzyme-catalyzed kinetic resolutions [2,13]. (±)-4-Phenylbutan-2-amine (*rac*-**1b**) contains a conformationally more flexible aralkyl substituent, whereas in (±)-1,2,3,4-tetrahydro-1-naphthalenamine (*rac*-**1c**) the amino group is attached to a conformationally rigid saturated ring. The amines *rac*-**1a–c** each representing different structural types proved to be versatile test substrates for the screening of the biocatalytic properties of differently immobilized forms of CaLB in kinetic resolution (Fig. 1).

3.1. Comparison of the productivity and selectivity of CaLB biocatalysts in batch and continuous-flow mode kinetic resolutions of the amines (rac-**1a–c**)

First, the four immobilized CaLB biocatalysts (CaLB N435, CaLB G250P, CaLB T2-150 or CaLB SGA10D) were tested in the kinetic resolutions of the three selected racemic amines (*rac*-**1a–c**) in batch mode and continuous-flow mode at 30 °C (Table 1). The batch mode experiments were performed using shake flasks whereas continuous-flow reactions were carried out in heat and pressure resistant stainless steel columns (CatCart™) using a continuous-flow bench-top lab reactor system allowing precise control and variation of temperature (0–70 °C) (Fig. 1).

This comparison revealed that irrespective of the nature of the substrate or the mode of enzyme immobilization, the productivity (specific reaction rate, *r*) of the continuous-flow system always surpassed the corresponding value for batch mode (Table 1). This result was in agreement with our previous studies on kinetic resolutions of secondary alcohols with immobilized lipases in continuous-flow reactors [29,44,45,52]. Enhanced productivity of a given enzyme in a continuous-flow system compared to the corresponding batch reaction can be rationalized by the much lower (~34%) voidage in a packed bed immobilized enzyme reactor as compared to over 90% in a stirred tank (shake flask) reactor [29,53]. Productivity enhancement (r_{flow}/r_{batch}) was much more dependent on the mode of immobilization (1.1–1.6 for CaLB N435, 3.9–5.7 for CaLB G250P, 4.2–5.5 for CaLB T2-150 and 2.7–4.0 for CaLB SGA10D) than on the nature of the substrate. The fact that selectivity (*E*) in the continuous-flow system remained unaltered or even slightly increased as compared to the batch reaction was also in agreement with our previous data on kinetic resolutions of secondary alcohols in continuous-flow reactors [29,44,45,52].

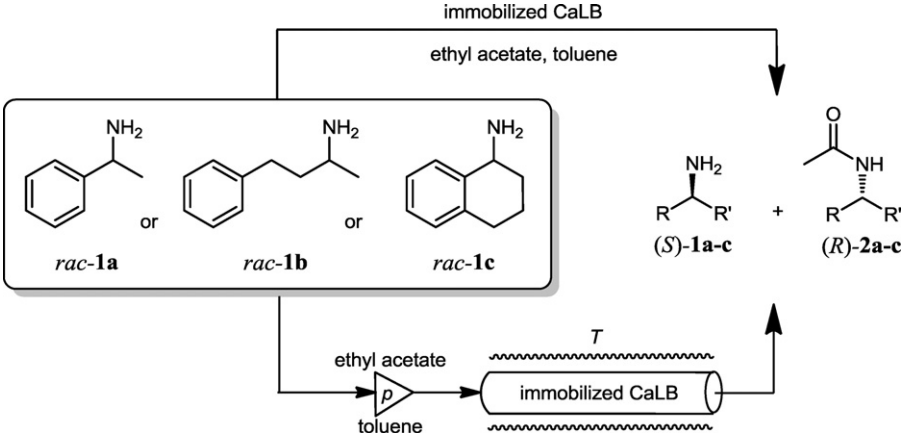

Fig. 1. Kinetic resolution of racemic amines *rac*-**1a–c** by acylation with ethyl acetate catalyzed by variously immobilized forms of lipase B from *Candida antarctica*.

Table 1
Kinetic resolution of the racemic amines *rac*-**1a–c** by acylation with ethyl acetate catalyzed by variously immobilized forms of lipase B from *Candida antarctica* at 30 °C in a shake flask (Panel A) or (Panel B) in a continuous-flow packed-bed reactor.

No.	Amine	CaLB	c (%)/t (h)	$ee_{(R)\text{-}\mathbf{2}}$ (%)	E[a]	r_{batch} (μmol min^{-1} g^{-1})
Panel A[b]						
1	*rac*-**1a**	N435	20.7/2	99.4	>200	7.1
2	*rac*-**1b**	N435	25.3/1	99.0	>200	14.1
3	*rac*-**1c**	N435	9.5/2	99.5	>200	2.7
4	*rac*-**1a**	G250P	19.1/6	99.3	>200	2.2
5	*rac*-**1b**	G250P	26.8/4	99.1	>200	3.7
6	*rac*-**1c**	G250P	4.8/4	98.8	>100	0.7
7	*rac*-**1a**	T2-150	8.1/6	98.5	>100	0.9
8	*rac*-**1b**	T2-150	12.5/4	99.3	>200	1.7
9	*rac*-**1c**	T2-150	2.1/6	94.8	38	0.2
10	*rac*-**1a**	SGA10D	5.6/4	99.0	>200	1.0
11	*rac*-**1b**	SGA10D	3.1/2	99.5	>200	0.9
12	*rac*-**1c**	SGA10D	0.4/2	95.0	39	0.1
No.	Amine	CaLB	c (%)	$ee_{(R)\text{-}\mathbf{2}}$ (%)	E[a]	r_{flow} (μmol min^{-1} g^{-1})
Panel B[c]						
13	*rac*-**1a**	N435	18.4	>99.9	≫200	11.3
14	*rac*-**1b**	N435	28.7	99.1	>200	14.3
15	*rac*-**1c**	N435	8.4	99.6	>200	4.3
16	*rac*-**1a**	G250P	18.0	>99.9	≫200	12.6
17	*rac*-**1b**	G250P	28.9	99.3	>200	16.5
18	*rac*-**1c**	G250P	4.5	99.8	≫200	2.7
19	*rac*-**1a**	T2-150	7.4	>99.9	≫200	4.8
20	*rac*-**1b**	T2-150	13.5	99.5	>200	7.2
21	*rac*-**1c**	T2-150	2.2	99.4	>200	1.1
22	*rac*-**1a**	SGA10D	4.9	>99.9	≫200	3.9
23	*rac*-**1b**	SGA10D	4.0	99.5	>200	2.4
24	*rac*-**1c**	SGA10D	0.5	96.0	50	0.4

[a] Enantiomeric ratio (E) was calculated from c and $ee_{(R)\text{-}\mathbf{2a–c}}$ [51]. For simplicity, E values calculated in the range of 100–200 were given as >100, those in the range of 200–500 as >200 and above 500 as ≫200.
[b] Batch reactions (see Section 2.4).
[c] Continuous-flow reactions at stationary state (see Section 2.5).

3.2. Temperature effects on the productivity and selectivity of CaLB biocatalysts in continuous-flow kinetic resolutions of the amines (*rac*-**1a–c**)

Decreasing selectivity at higher temperatures in amine acylation with CaLA [40] or the local maxima in kinetic resolutions of secondary alcohols by CaLB-catalyzed acylation [29] indicated already that the trends of temperature dependence were not uniform for the biocatalytic properties of lipases. However, to the best of our knowledge, no comparative data have been published on the temperature effects on lipases immobilized by different methods. Therefore, the major goal of our study was to investigate the temperature dependent behavior of various immobilized CaLB biocatalysts. For this study, the continuous-flow reaction system was chosen due to its higher efficiency, easy control and good reproducibility as compared to the batch mode.

First, the typical substrate (±)-1-phenylethanamine (*rac*-**1a**) was tested in continuous-flow reactors filled with differently immobilized CaLBs at temperatures 0–70 °C (in 10 °C steps) (Fig. 2). Because ethyl acetate in dry toluene proved to be a suitable acylating agent in CaLB-catalyzed kinetic resolutions of *rac*-**1a** [2,13], our experiments were performed using the same system in small stainless-steel columns (0.82 mL inner volume) filled with immobilized CaLBs (0.21–0.29 g) working at a fixed flow rate (0.2 mL min^{-1}). The results with *rac*-**1a** have indicated already that the mode of immobilization was a significant factor concerning the properties of the biocatalyst. The two CaLB preparations immobilized by physical adsorption (N435 and G250P) behaved quite similarly: between 0 and 70 °C the productivity (specific reaction rate, r_{flow}) increased almost linearly whereas above 40 °C the enantiomeric excess of the product ($ee_{(R)\text{-}\mathbf{2a}}$) and the degree of the enantiomeric ratio (E) dropped off ($ee_{(R)\text{-}\mathbf{2a}}$ decreased from >99.9% to about 99.4% and E values from ∼3000 to ∼600). Covalently bound CaLB (T2-150) showed similar temperature dependence. After a short hyperbolic ascending section (0–20 °C), productivity increased linearly between 20 and 70 °C. On the other hand, decline of both enantiomeric excess ($ee_{(R)\text{-}\mathbf{2a}}$) and enantiomeric ratio (E) in the 40–70 °C range was less pronounced as compared to physically adsorbed CaLBs (N435 and G250P). The sol–gel-entrapped CaLB (SGA10D) behaved rather differently than the other forms of CaLB. It was almost inactive at low temperatures (0–10 °C) but after a hyperbolic ascending section between 20 and 70 °C its productivity (r_{flow}) almost reached that of physically adsorbed CaLBs. Note that among the four CaLBs tested SGA10D was the conformationally most restricted form and in the kinetic resolution of *rac*-**1a** it suffered the smallest loss of selectivity at higher temperatures. Thus, whereas at 40 °C it exhibited the lowest enantiomeric excess ($ee_{(R)\text{-}\mathbf{2a}}$) and enantiomeric ratio (E), at 70 °C the $ee_{(R)\text{-}\mathbf{2a}}$ and E values of this entrapped enzyme remained the highest.

The temperature dependant trends in the biocatalytic properties of immobilized CaLBs with the conformationally more flexible (±)-4-phenylbutan-2-amine (*rac*-**1b**) were similar but more pronounced than the ones found with *rac*-**1a** between 0 and 70 °C (Fig. 3). In the kinetic resolutions of *rac*-**1a** in continuous-flow reactors, hyperbolically descending increase of productivity (specific reaction rate, r_{flow}) was observed both for physically adsorbed (N435 and G250P) and covalently bound (T2-150) CaLBs, whereas a hyperbolically ascending increase of r_{flow} was found for the entrapped CaLB SGA10D. Similarly to the kinetic resolution of *rac*-**1a**, all the immobilized CaLBs exhibited a monotonous decrease of enantiomeric excess ($ee_{(R)\text{-}\mathbf{2b}}$) and enantiomeric ratio (E) between 0 and 70 °C in acetylation of *rac*-**1b** as well. The conformationally most restricted sol–gel-entrapped form of CaLB (SGA10D) was the least selective at temperatures up to 50 °C while with this form $ee_{(R)\text{-}\mathbf{2b}}$ and E reached a maximum above 50 °C with this form.

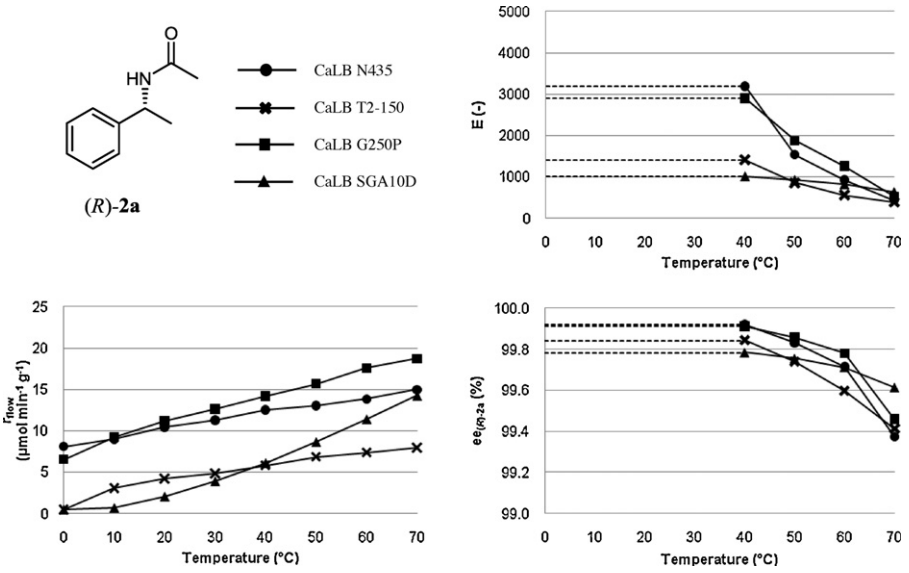

Fig. 2. Temperature dependent biocatalytic properties (r_{flow}, E and $ee_{(R)\text{-}\mathbf{2a}}$) of variously immobilized CaLB biocatalysts in kinetic resolution of (±)-1-phenylethanamine (*rac*-**1a**) by acylation with ethyl acetate in continuous-flow reactors.

The most complex changes in the biocatalytic properties of immobilized CaLBs between 0 and 70 °C were observed in the continuous-flow kinetic resolutions of the conformationally least flexible model, i.e. (±)-1,2,3,4-tetrahydro-1-naphthalenamine (*rac*-**1c**) (Fig. 4). Productivity (specific reaction rate, r_{flow}) change in acylation of *rac*-**1c** followed a sigmoid curve for the physically adsorbed CaLBs (N435 and G250P) providing more flexibility for the enzyme whereas a hyperbolically ascending increase of r_{flow} was found for the covalently bound and entrapped CaLBs (T2-150 and SGA10D, respectively) representing conformationally more restricted forms of the enzyme. The role of substrate and enzyme flexibility in the selectivity of the enzyme reactions was even more significant with the rigid substrate *rac*-**1c**. Again, the physically adsorbed, conformationally most flexible CaLBs (N435 and G250P) behaved similarly and showed selectivity maxima at around 50 °C (see curves for $ee_{(R)\text{-}\mathbf{2c}}$ and E in Fig. 4). Conformational restrictions of the enzyme in the covalently bound CaLB (T2-150) in the kinetic resolution of the relatively rigid *rac*-**1c** resulted in significant decrease in selectivity (e.g. $ee = 93.2\%$ and $E = 29$ at 0 °C) compared to the adsorbed and therefore less restricted CaLBs ($ee = 99.1\%$ and $E = 240$; and $ee = 99.1\%$ and $E = 230$ for N435 and G250P at 0 °C, respectively). However, the nature of the selectivity change as a function of temperature remained similar with a maximum ($ee = 99.7\%$ and $E = 840$) at around 50 °C. The most dramatic difference was observed when the kinetic resolution of the rigid *rac*-**1c** was performed with the conformationally most restricted sol–gel-entrapped CaLB (SGA10D). At low temperature, this version of immobilization resulted in almost negligible activity and selectivity (e.g. $r_{flow} = 0.03\ \mu mol\ min^{-1}\ g^{-1}$, $ee = 37.8\%$ and $E = 2.2$ at 0 °C). Selectivity with the rigid entrapped CaLB SGA10D showed also a maximum but at higher temperature ($ee = 99.1\%$ and $E = 220$ at 60 °C) and remained quite low as compared to the conformationally less restricted other CaLBs.

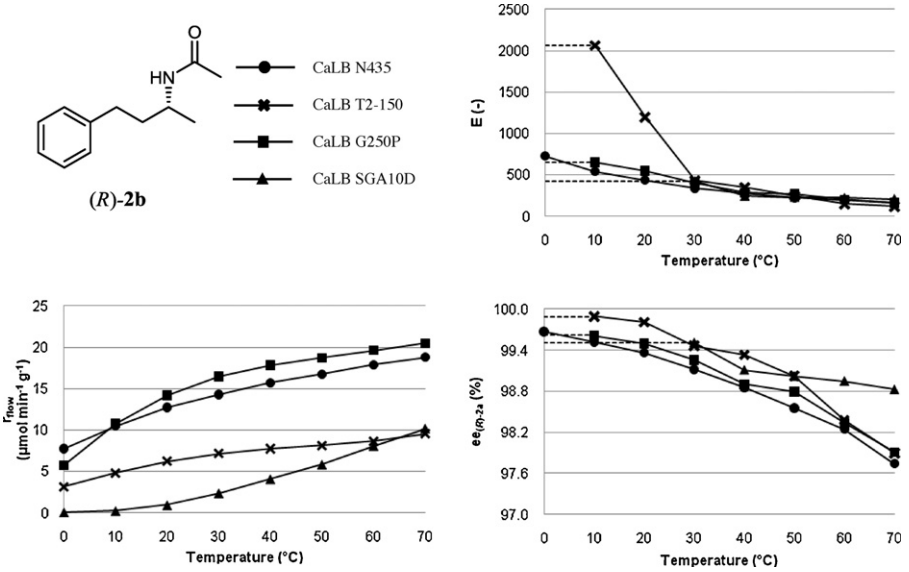

Fig. 3. Temperature dependent biocatalytic properties (r_{flow}, E and $ee_{(R)\text{-}\mathbf{2b}}$) of variously immobilized CaLB biocatalysts in kinetic resolution of (±)-4-phenylbutan-2-amine (*rac*-**1b**) by acylation with ethyl acetate in continuous-flow reactors.

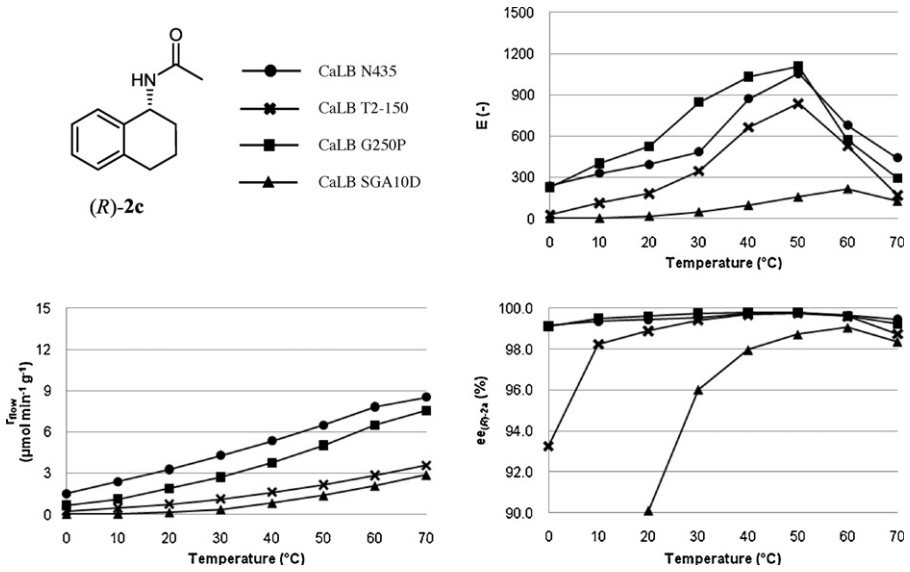

Fig. 4. Temperature dependent biocatalytic properties (r_{flow}, E and $ee_{(R)\text{-}\mathbf{2c}}$) of variously immobilized CaLB biocatalysts in kinetic resolution of (±)-1,2,3,4-tetrahydro-1-naphthalenamine (*rac*-**1c**) by acylation with ethyl acetate in continuous-flow reactors.

*3.3. Rationalization of the effect of temperature on productivity and selectivity of the different CaLB biocatalysts toward the various types of amines (rac-**1a–c**)*

To the best of our knowledge, the present one is the first systematic study which provided comparative data for temperature dependent biocatalytic properties of a lipase immobilized by different methods involving substrates of various conformational flexibility. Our data demonstrated the essential role of conformational characteristics of both substrate and enzyme in a stereoselective enzyme reaction. In general, decreasing flexibility of the enzyme (owing to covalent attachment or entrapment in a sol–gel) seems to be beneficial for selectivity with conformationally flexible substrates. Contrarily, for non-flexible substrates decreased flexibility of the enzyme diminishes both productivity and selectivity.

The fact that enantioselectivity of the enzyme depended significantly on the mode of immobilization [46,47] indicated that enzyme may have different conformation induced by the interactions with the support because different areas of the protein may be involved in the immobilization. In addition, the mode of immobilization (i.e. adsorption, covalent binding or entrapment) can influence the mobility of various enzyme regions differently. Temperature can affect the physical–chemical behavior of the reactions (without calling for direct enzymatic effect) such as partition of water, adsorption of products and substrates. The immobilized enzyme system is basically a multiphase environment where the support can affect the reaction in a similar way as solvents affect the reaction of the non-immobilized enzyme. Most of these effects influence the productivity and the selectivity of the immobilized enzyme.

Our results may be rationalized – at least partially – on the basis of the "induced fit" theory [54]. Enzyme specificity is linked to conformational differences between the reactive complex formed with a good substrate and the related complex with a poor one [55]. It was shown that a conformational change may act as a molecular switch to select the right substrate and to recognize and disfavor the reaction with an incorrect substrate [56]. Recent data obtained by experimental methods and computation increasingly reveal that conformational dynamics are essential factors in enzyme functions throughout the catalytic cycle [57]. Conformational transitions in each step of the catalytic cycle (substrate recruiting, chemical transformation, and product release) may involve conformational selection and induced fit (which can be viewed as a special case in the catalytic network) modulating enzyme specificity and efficiency [57].

Analysis of the conformational stability and activity of CaLB by computation indicated that the overall conformation of CaLB is stable in the organic solvents and the conformational change in the region of the active site is the main factor influencing the activity [58]. High level calculations on the CaLB-catalyzed transacylation of methyl acrylate derivatives compared with the experimentally determined acrylation rates demonstrated that substrate conformations may set the rate of biotransformation [59].

Optical spectroscopic methods (CD and DRIFT) indicated that the secondary structure of subtilisin and CaLB was nearly identical in free and in immobilized state (adsorption onto solid supports) [60]. Another study on the structural dynamics of a Cu, Zn superoxide dismutase by various spectroscopic methods showed that the sol–gel entrapped enzyme preserved its structural integrity and functionality, while below the isoelectric point the global motion of the protein was totally hindered [61].

The high level of selectivity achieved at lower temperatures with conformationally flexible substrates **1a**,**b** indicates that conformation populations for the faster reacting enantiomers and the corresponding transition states and products [i.e. transition from (*R*)-**1a**,**b** to (*R*)-**2a**,**b**] are different enough from those of the slower reacting enantiomers [(*S*)-**1a**,**b**]. The significant loss of selectivity at elevated temperatures may be rationalized – at least partially – by the enhanced mobility of the enzyme resulting in decreased difference between the conformation populations of the (*R*)-**1a**,**b** and (*S*)-**1a**,**b** related states. On the other hand, sol–gel entrapment restricts enzyme mobility even at elevated temperature resulting in more preserved selectivity with such flexible substrates.

The behavior of CaLBs immobilized by various methods with the conformationally restricted substrate (±)-**1c** can also be rationalized on this basis. "Induced-fit" [54–57] can result in conformation populations of the mobile forms of the enzyme to better accommodate the fast reacting enantiomer (*R*)-**1c**, its transition state and product [i.e. to prefer transition from (*R*)-**1c** to (*R*)-**2c**] than those of the less matching enantiomer (*S*)-**1c**. Therefore, the conformationally non-restricted forms of CaLB can exhibit relatively high selectivity toward the enantiomers of rigid **1c**. If enzyme mobility is restricted also (as with CaLB T2-150 and especially with CaLB

SGA10D), the "induced-fit" like behavior of the enzyme enabling reactivity and selectivity approaches the rigid "key–lock" situation. Such rigid "key–lock" case with poor match of either enantiomers results in low reactivity and selectivity. With increasing temperature the enhanced mobility of the enzyme allows better matches and situation shifts toward "induced-fit" like behavior enabling higher activity and selectivity.

4. Conclusions

Our results have demonstrated for the first time that in lipase-catalyzed kinetic resolutions of racemic amines for different kinds of substrates or reaction conditions different modes of enzyme immobilization were optimal. The various modes of immobilization (physical adsorption, covalent binding or sol–gel entrapment) of *C. antarctica* lipase B influenced significantly the nature of the temperature effect (in the range of 0–70 °C) on enzyme productivity (r_{flow}), enantiomeric excess of the products ($ee_{(R)\text{-}\mathbf{2a–c}}$) as well as enantiomeric ratio (E) in continuous-flow kinetic resolution of the racemic amines *rac*-**1a–c**. This behavior may be associated with altered enzyme flexibility due to the various immobilization modes. A proper combination of enzyme and substrate flexibility seemed to have profound effect on the properties (r_{flow}, $ee_{(R)\text{-}\mathbf{2a–c}}$ and E) of the biocatalytic process. Immobilization methods restricting enzyme mobility in combination with flexible substrates were beneficial for preserving selectivity at elevated temperatures. On the other hand, immobilization methods restricting enzyme mobility in combination with rigid substrates resulted in poor reactivity and selectivity at low temperature.

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