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The amidase activity of *Candida antarctica* lipase B is dependent on specific structural features of the substrates

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Abstract

The hydrolytic activity of *Candida antarctica* lipase B (**CAL-B**) was studied using 15 amides with different linear saturated acyl residues and substituents in the aromatic amine. A strong dependence of the hydrolysis rate on the length of the acyl residue and the substituents groups in the aromatic ring of the amides was demonstrated, with the highest hydrolytic initial reaction rates found for the C_{10} acyl derivates and benzylamides. The C_{10} benzylamide, an amide without substituents in the aromatic ring was hydrolyzed almost as fast as capsaicin and five times faster that the corresponding C_{10} vanillyl derivative. Therefore, a benzylamide bearing the non-linear unsaturated acyl residue of capsaicin (8-methyl-6-nonanenoic acid) was synthesized. This substrate was hydrolyzed four times faster than capsaicin. Although it has been widely claimed that lipases rarely display amidase activity, with this contribution we demonstrate that the amidase activity of **CAL-B** is dependent on the structural features of the substrate.

Keywords: Lipase; Amide hydrolysis; Benzylamides; 3-Methoxybenzylamides vanillylamides; *Candida antarctica*

1. Introduction

Enzymes have been increasingly applied to the synthesis of a wide variety of organic compounds replacing traditional chemical processes, particularly due to their enantio-, chemo- and regio-selective properties. Among the commercially available biocatalysts, those based on lipases are one of the most frequently used, because of the remarkable catalytic versatility of these enzymes. Lipases and particularly **CAL-B** have been used in the resolution and preparation of chiral alcohols, carboxylic acids, esters, amides and lactones, via synthesis, hydrolysis, alcoholysis, acidolysis or transesterification reactions [1–3]. In recent years, particular attention has been given to the resolution of chiral amines for the preparation of chiral building blocks [4]. However, the lack of amidase activity in lipases [2,5–8], does not allow the biocatalytic effective resolution of amines via a sequential amide synthesis–hydrolysis process. In general, low efficiencies have been obtained in the few reports where the hydrolysis of chiral amides has been attempted with lipases and subtilisin [8,9]. In order to cope with this limitation, various strategies have been proposed.

It is well known that the catalytic mechanism of lipases acting on an acylated substrate is undertaken through the formation of an acyl-enzyme intermediate that facilitates the nucleophilic attack. Thus, hydrolysis of amides in the presence of **CAL-B** has been achieved when the acyl-enzyme complex formation is induced either through substrate activation, as already reported for amides with 2-methoxyacetic acid derivatives [9], or with particular substrate structures such as amides of medium or long-chain acyl residues [10] (Scheme 1). In the same context, it has been demonstrated that *p*-substitution increases the hydrolysis of 1-phenyl-ethylamides (Scheme 1) [11].

An alternative strategy consists in the modification of the enzyme through protein engineering, as recently reported by Fujii et al., 2005 [12]. These authors constructed a double mutant of a lipase from *Pseudomonas aeruginosa* that gave a 2-fold increase in relative amidase activity when compared to that of the wild-type enzyme.

In order to explore the influence of substrate structural features on the feasibility of amide hydrolysis by lipases and to extend the range of amides suitable for lipase hydrolysis, in this

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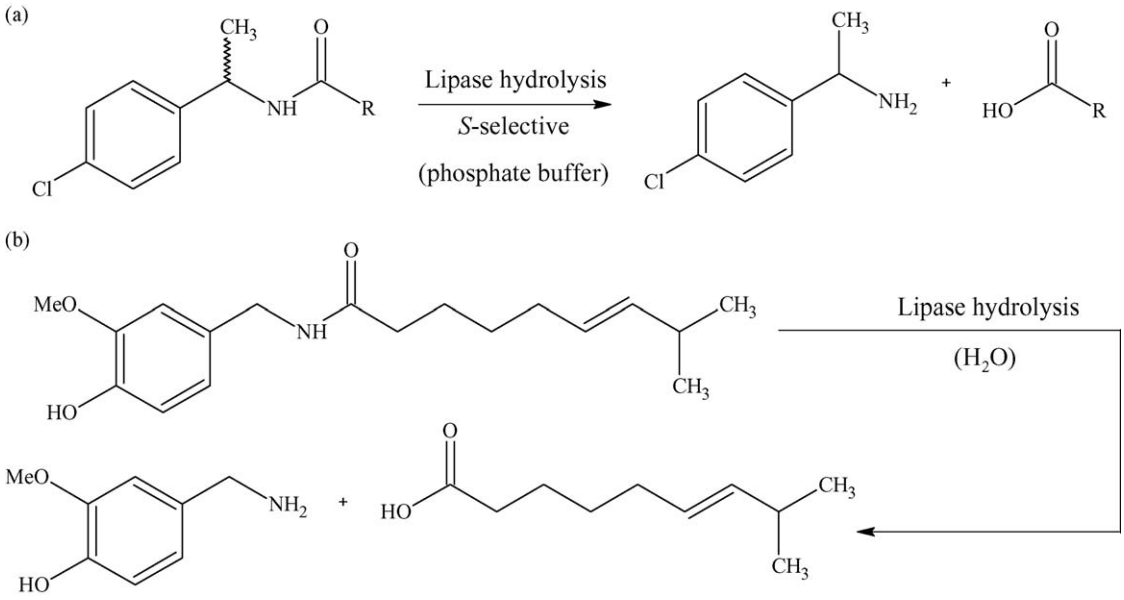


Scheme 1. (a) Use of 2-methoxyacetic acid derivate and *p*-chloro substituted phenylethylamine to enhance the hydrolytic activity in the lipase-catalyzed amide hydrolysis, from literature R = CH_2OCH_3 [9], R = CH_3 [11]. (b) Lipase-catalyzed hydrolysis of long acyl-chain amides (capsaicin) [10].

report we explore the effect of fatty acid chain length and the chemical nature of various arylamides on their susceptibility of hydrolysis by **CAL-B**.

2. Materials and methods

2.1. Materials

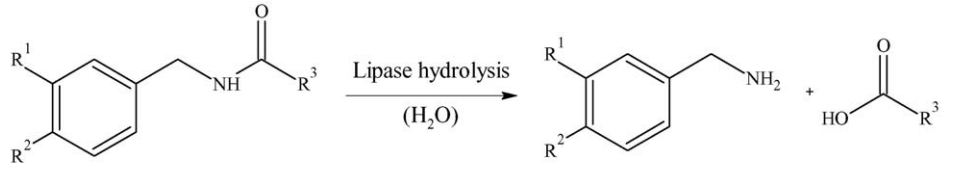
Immobilized lipase B from *Candida antarctica* (Novozym 435 **CAL-B**) was kindly supplied by Novozymes A/C (México city, Mexico). 2-Methyl-2-butanol (99.7%) methanol and acetonitrile (HPLC grade) were purchased from J.T. Backer (Edo. de México, Mexico). Vanillylamine hydrochloride (98%), 3-methoxybenzylamine (98%), capric acid (99%), ethyl butyrate (99%), ethyl caprylate (99%), molecular sieves (5 Å beads, 8–12 mesh), vanillylalcohol (98%) and *N,N*-diisopropylethylamine (DIPEA) were from Aldrich (WI, USA). Benzylamine (99%), capsaicin (97%), methyl caproate (99%), lauric (99%), myristic (99%) and palmitic (99%) acids were purchased from Sigma Chemical Co. (MO, USA).

2.2. Amide synthesis

Synthesis of amides reported in Scheme 2 were carried out in 5 ml sealed vessels at 45 °C agitated with magnetic stirring at equimolar ratio of the amine and acyl-donor (20 mM) in 2-methyl-2-butanol, fully dehydrated with molecular sieves.

In the synthesis of vainillylamides a tertiary amine (DIPEA) and vanillylamine hydrochloride (VAM·HCL) in a molar ratio of 18:1 were pre-incubated for 30 min to release vanillylamine. Reactions were started with the addition of 20 mg/ml of Novozym 435 as biocatalyst. In order to control the water activity (A_w) during the reaction, 20 mg/ml of molecular sieves were added to the reaction vessels. The structural analysis and purification process of all amides are described elsewhere [13].

For the synthesis of (*E*)-*N*-benzyl-8-methyl-6-nonenamide, an esterification reaction between vanillylalcohol and capsaicin (30:15 mM) was first carried out in 10 ml sealed vessels at 45 °C agitated with magnetic stirring in *n*-hexane fully dehydrated with molecular sieves. A yield of 48% of capsaite (the ester analogue of capsaicin) was obtained and purified by flash chromatography



1: R^1 = -OMe R^2 = -OH	a: R^3 = $-CH_2CH_2CH_3$ b: R^3 = $-CH_2(CH_2)_3CH_3$
2: R^1 = -OMe R^2 = -H	c: R^3 = $-CH_2(CH_2)_5CH_3$ d: R^3 = $-CH_2(CH_2)_7CH_3$
3: R^1 = -H R^2 = -H	e: R^3 = $-CH_2(CH_2)_9CH_3$ f: R^3 = $-CH_2(CH_2)_{11}CH_3$
	g: R^3 = $-CH_2(CH_2)_{13}CH_3$ h: R^3 = $-CH_2(CH_2)_3CH{=}CHCH_2(CH_3)_2$

Scheme 2. Nature of amine substituents and fatty acid residues considered for hydrolysis by *Candida antarctica* lipase B.

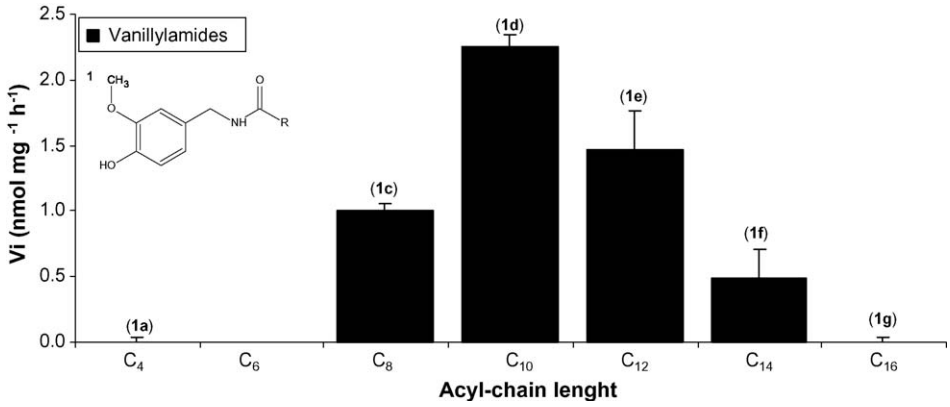


Fig. 1. Effect of the acyl-chain length on the initial reaction rate of the lipase-catalyzed vanillylamide hydrolysis.

(*n*-hexane/ethyl acetate 70:30). The purified capsiate was then subjected to an aminolysis reaction with benzylamine in 10 ml sealed vessels at 45 °C with magnetic stirring, using an equimolar ratio of capsiate and benzylamine (15 mM) in 2-methyl-2-butanol fully dehydrated with molecular sieves. A 99% yield of (*E*)-*N*-benzyl-8-methyl-6-nonenamide was obtained.

2.3. Amide hydrolysis

All hydrolysis reactions were carried out in 5 ml sealed vessels at 70 °C with magnetic stirring. The total amide concentration was 1.5 mM in deionized water at pH 7.5 forming bifasic mixtures. The reaction was started by the addition of 6 mg/ml of the enzyme. Samples of 50 μl were withdrawn at regular intervals during 8 h and centrifuged (16,000 × *g* × 3 min) to separate the enzyme; the products were finally analyzed by HPLC.

2.4. HPLC analysis

Reaction products were quantified by HPLC using a Waters 600E system controller (Waters Corp., Milford, MA) with a flow rate of 1 ml min^{-1} equipped with a Waters 996 photodiode array detector. Vanillylamine and 3-methoxybenzylamine were separated with a Waters Spherisorb ODS-2 column (4.6 mm × 250 mm, Waters Corp., Milford, MA). Vanillylamine was eluted with a solvent mixture of 50:50 (v/v) methanol–water and detected at 230 nm (3.5 min). 3-Methoxybenzylamine was eluted with a solvent mixture of 70:30 (v/v) acetonitrile–water and detected at 275 nm (4.0 min). Benzylamine was separated with a Supelcosil C_{18} column (3.9 mm × 300 mm, Supelco, Bellefonte, PA) operating at a solvent mixture of 70:30 (v/v) acetonitrile–phosphate buffer (25 mM, pH 4.2) and detected at 206 nm (5.1 min).

3. Results and discussion

3.1. Effect of the fatty acid chain length on the hydrolysis of vanillylamides

The initial reaction rate of hydrolysis of different vanillylamides containing acyl residues C_4 and C_8–C_{16} (**1a and 1c–g**, Scheme 2), was determined in similar conditions to those previously reported for capsaicin hydrolysis: 1.5 mM of substrate and 6 mg/ml of enzyme at 70 °C in 5 ml of deionized water [10]. As shown in Fig. 1, only medium acyl-chain length amides (C_8–C_{14} **1c–f**) were hydrolyzed at significant reaction rates, with the highest value found for the C_{10} acyl amide (**1d**, 2.3 nmol mg^{-1} h^{-1}). In the case of esters, previous reports point out that the catalytic efficiency of **CAL-B** is enhanced with medium and long-chain acyl donors [14–16]. The vanillylamides containing C_4 and C_{16} acyl residues (**1a** and **1g**) were not hydrolyzed after 4 days. These results are consistent with previous reports, where low hydrolysis rates were obtained with short-chain amides (C_2 or C_4), requiring 2-methoxyacetic acids and chlorophenyl derivatives for the improvement of the hydrolysis reaction rate [9]. In general, it has been suggested that lipase substrate specificity is the consequence of the length and shape of the binding site of lipases, with steric hindrance increasing for long-chain fatty acids. For the short length fatty acids, specific interactions are significantly lower, resulting in lower reaction rates.

3.2. Hydrolysis of aromatic amides containing different substituents on the aryl ring

In order to explore the influence of the substituent group in the vanillylamine, hydrolysis of 3-methoxybenzyl- and benzylamides containing acyl-chains of different lengths (**2b–e** and **3b–e**, respectively) was carried out in similar conditions as those described above. These results are shown in Fig. 2 where it is interestingly to observe that a similar acyl-chain length activity profile as the one already described with vanillylamides was obtained, with the highest reaction rates also found for the C_{10} 3-methoxybenzyl-(**2d**, 5.2 nmol mg^{-1} h^{-1}) and benzylamide (**3d**, 11.3 nmol mg^{-1} h^{-1}). Comparing the hydrolysis reaction rate of the C_{10} amides **2d** and **3d** with the hydrolysis rate obtained for the corresponding vanillylamide **1d**, it may be concluded that the absence of substitutions in the benzyl ring increases the lipase specificity as the hydrolysis rate increases 2.3- and 5-fold, respectively.

A similar behaviour has already been reported for various substituents of the phenyl ring in the hydrolysis of butanoates

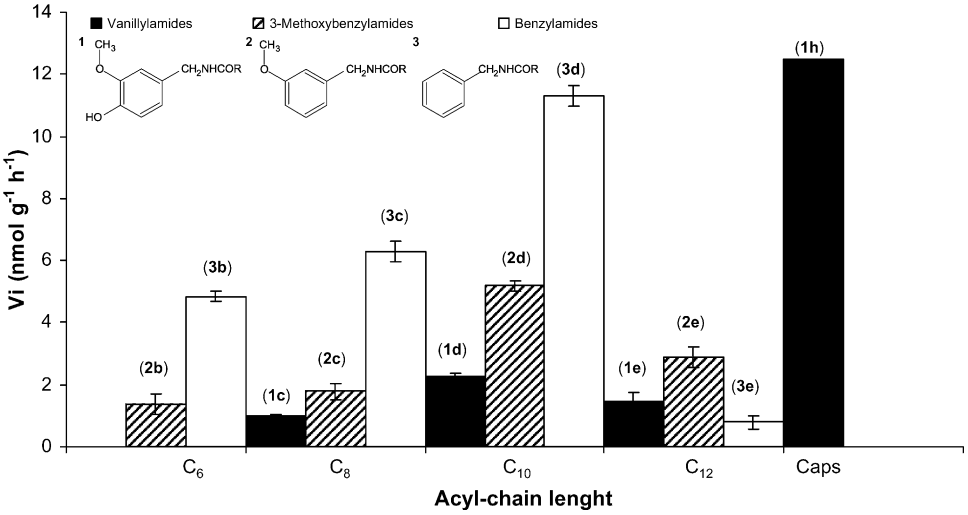


Fig. 2. Effect of the acyl-chain length and leaving group in the initial rate of the lipase-catalyzed vanillylamide hydrolysis. Caps (**1h**): initial reaction rate of capsaicin from literature [10].

of *o*-, *m*- or *p*-substituted phenols, where the rate differences are more evident due to the stronger inductive effect of the substituted phenyl ring [17]. Nevertheless, it may be also possible that the differences arise from the interaction of the aryl ring substituent with the active site [11,17,18]. In fact, in a previous report we have demonstrated a similar preference profile in the lipase-catalyzed synthesis of the vanillyl-3-methoxybenzyl- and benzylamides containing same acyl-chains, where the higher initial reaction rates were observed for benzylamides, intermediate rates for 3-methoxybenzylamides and the lowest rates for vanillylamides [13].

Despite the higher hydrolysis rate found for C_{10} benzylamide (**3d**), it was not possible to reach the capsaicin (**1h**) hydrolysis rate. As the only difference is the unsaturated and branched nature of the acyl group (8-methyl-6-nonanenoic acid), we decided to explore the specificity of **CAL-B** towards an amide bearing the benzyl ring and this acyl residue. As expected, the hydrolysis rate of this new amide (**3h**, 45.6 nmol mg^{-1} h^{-1}) was notably higher than the hydrolysis rate of capsaicin (**1h**, 12.5 nmol mg^{-1} h^{-1}) (3.7-fold), as shown in Fig. 3.

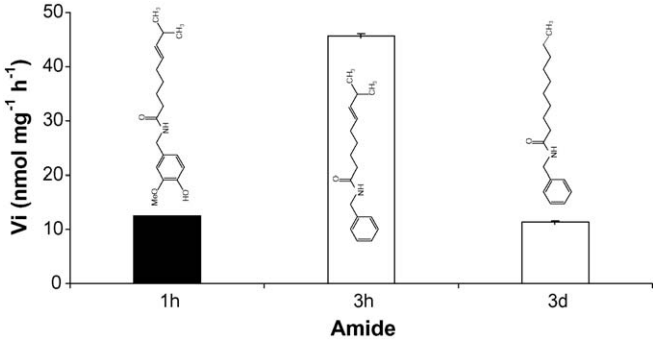


Fig. 3. Increase on the initial reaction rate in the lipase-catalyzed benzylamide (**3h**) hydrolysis by the combined favourable effect of the branched unsaturated acyl residue and benzylamine. Caps (**1h**): initial reaction rate of capsaicin from literature [10].

4. Conclusions

Sixteen different amides were synthesized and subjected to hydrolysis with lipase B from *C. antarctica*. It was established that the efficiency in terms of reaction rate of this process is directly related to the structural features of the substrate. Particularly, the strict dependence of the hydrolysis rate on the length of the acyl residue of the amides was demonstrated. Indeed, in terms of amides containing linear saturated fatty acids, the highest initial reaction rates of hydrolysis were obtained with the C_{10} acyl derivatives (**1**–**3d**). A strong influence of the subtituents in the amide aryl ring on the catalytic behaviour of lipases was also observed, the highest rate obtained for benzylamides and the lowest for vanillylamides. Despite the combination of these two optimal structural features in C_{10} benzylamide (**3d**), it was hydrolyzed at lower rate than capsaicin (**1h**). The hydrolysis of an amide containing the non-linear unsaturated acyl residue of capsaicin (8-methyl-6-nonanenoic acid) and the benzyl aryl ring was 3.7 times faster than capsaicin and four times faster than the corresponding hydrolysis rate of the C_{10} benzyl derivative **3d**. In summary, we have demonstrated that amide hydrolysis by **CAL-B** strongly depends on substrate structural features. The use of this strategy opens the possibility to increase the impact of lipases in organic synthetic processes as an optimum amine or acyle derivative may be chosen for the effective resolution of a given chiral amine.

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