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Effect of the immobilization protocol in the activity, stability, and enantioslectivity of Lecitase® Ultra

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Abstract

Lecitase® Ultra is a commercial enzyme preparation designed for degumming of oils. In this paper, we show that this enzyme increased its hydrolytic activity in the presence of low concentrations of detergents in hydrolytic reactions (e.g., 150-fold using 0.01% hexadecyltrimethylammonium bromide). Moreover, the enzyme becomes adsorbed on octyl agarose at low ionic strength, increasing the activity by a 13-fold factor. The enzyme adsorbed on octyl-agarose was desorbed by addition of detergents and immobilized on supports coated with polyethyleneimine (PEI) and in cyanogen bromide agarose. This immobilized preparations exhibited very different activity and enantioselectivity in the hydrolysis of (±)-methyl mandelate and (±)-2-*O*-butanoyl-2-phenylacetic acid. For example, the enzyme immobilized on octyl agarose yielded R-mandelic acid with an *E* value higher than 100, while the CNBr preparation gave an *E* value of 26, favoring the S isomer. The immobilized enzyme was quite stable at pH 5 and 7, and in the presence of organic solvents.

Thus, Lecitase® Ultra seems to have very good prospects to be used as enantioselective biocatalyst in fine chemistry.

Keywords: Lipases; Phospholipases; Resolution of racemic mixtures; Hyperactivation by detergents; Interfacial adsorption; Mandelic acid

1. Introduction

Phospholipases A_1 hydrolyze the 1-acyl group of a phospholipid to lysophospholipid and fatty acid. In mammals, these enzymes may play a relevant role in both phospholipidosis [1] and virulence factors for bacterial and fungal pathogenesis [2]. Moreover, phospholipases A_1 are of particular interest for industrial applications, to produce 2-acyl-lysophospholipids (good emulsioners) with interesting fatty acid composition (eicosapentaenoic acid, conjugated linoleic acid, and docosahexaenoic acid) and degumming process of oils [3]. The interest on finding new phospholipases is shown by the high number of sources for this enzyme discovered in recent years [4]. Availability and stability are also requirements to take full advantage of these enzymes. Thus, for example, to increase the range of applications of these enzymes, the stability of a phospholipase A1 from *Serratia* sp in organic solvents by directed evolution has been tested [5]. Recently, a new enzyme preparation with phospholipase A1 activity, namely Lecitase® Ultra, was patented [6] and made commercially available. Following the supplier information, this is a preparation obtained from the fusion of the genes of the lipase from *Thermomyces lanuginosus* and the phospholipase from *Fusarium oxysporum* and developed principally for degumming processes [6]. This new enzyme presented the stability of the lipase from *T. lanuginosus* and the activity of the enzyme from *F. oxysporum* [6]. However, it also has biocatalytic properties that are useful in the synthesis of tailored phospholipids, although due to the relative novelty of this enzyme product only few manuscripts may be found in literature on its uses [7].

Phospholipases have been described to undergo interfacial activation [8], similarly to lipases [9]. Lipases present a broad specificity combined with a high region and enantioselectivity, and may be used in many different reaction media. However, to our knowledge, no attempts have been performed to use Lecitase® Ultra as biocatalyst for fine chemistry, using substrates far from phospholipids (e.g., using the enzyme preparation to resolve racemic mixtures of chiral compounds).

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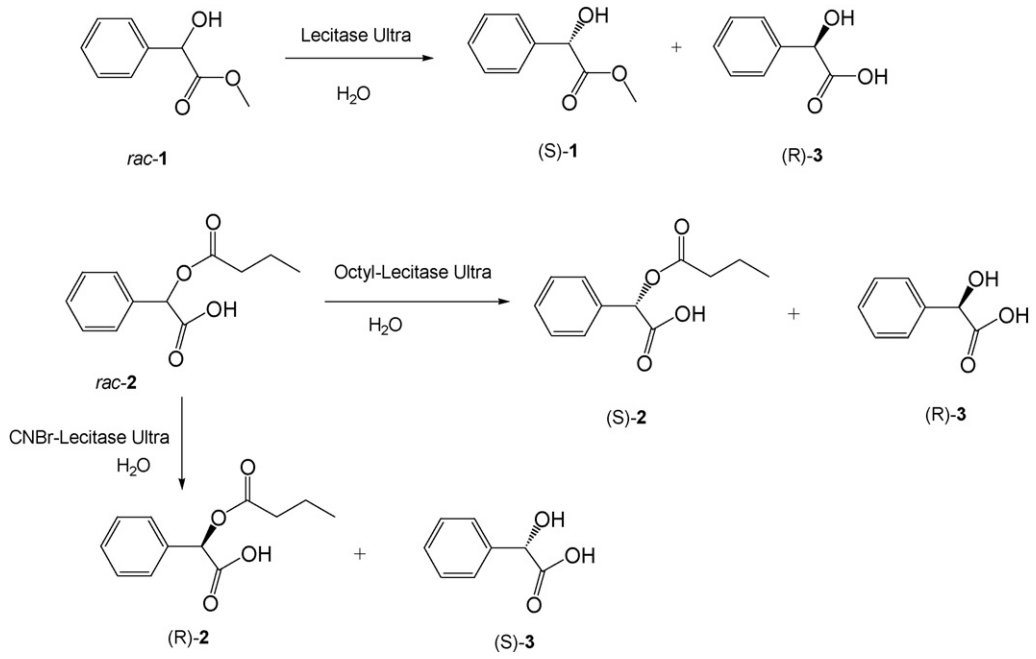

Scheme 1. Hydrolytic resolution of mandelic acid derivatives by immobilized lipases.

We intend to find if the similarities of Lecitase® Ultra to lipases may be extended to some other characteristics useful for their industrial implementation as biocatalysts in the enantioselective hydrolysis of racemic mixtures of esters. First, we will study the possibilities of this enzymatic preparation to become interfacially activated by adsorption on hydrophobic supports at low ionic strength, like octyl-agarose. This support has been described to be useful for the one-step purification, immobilization, and hyperactivation of lipases [10], because it is able to immobilize the open form of the lipase *via* interfacial activation [11]. Moreover, it has been described that lipase activity may be increased and modulated by the addition of small concentrations of different detergents, improving their performance in biotransformations [12]. We will study if that positive effect of detergents on lipase activity may also be detected using Lecitase® Ultra.

Moreover, we will investigate if Lecitase® Ultra may be a potentially useful biocatalyst in the resolution of racemic mixtures. As a model substrate, we have used different mandelic acid derivatives: (±)-methyl mandelate [(±)-**1**], presenting the stero center in the acyl donor and (±)-2-*O*-butanoyol-2-phenylacetic acid [(±)-**2**], presenting the stero center in the nucleophile (Scheme 1). The pure isomers of these compounds present some interest. For example, R-mandelic acid [R-(**3**)] isomer is used in the production of Cefamandole [13], Cefonicid [14], (+)-goniodiol (1′R,2′S,5R)-5-(1′,2′-dihydroxy-2-phenylethyl)-pent-2-eno-5-lactone [15], styryllactones[16] or 1-aza-cryptophycin [17]. [S-(**3**)] may be used in the production of 1-methyl-7-oxabicyclo[2.2.1]heptan-2-one [18], (-)-8-epi-9-deoxygoniopypyrone [19], etc.

In these studies, we will use immobilized enzymes, firstly to avoid the problem of enzyme dimerization that could alter the lipase properties [20], and secondly to study the possibilities of modulating the properties of the Lecitase® Ultra by using different immobilization strategies as previously described for many standard lipases [21].

2. Experimental

2.1. Materials

Lecitase® Ultra was obtained from Novozymes (Denmark). Agarose 6BCL, octyl-agarose 4BCL and cyanogen bromide agarose (CNBr-activated Sepharose 4BCL) were purchased from Pharmacia Biotech (Uppsala, Sweden). Polyethyleneimine (PEI) (Mr 25000), Triton X-100, hexadecyltrimethylammonium bromide (CTAB), *p*-nitrophenyl butyrate (pNPB) were from Sigma. Glyoxyl-agarose [22], and PEI-agarose [23] were prepared as previously described. (±)-α-Hydroxy-phenylacetic acid methyl ester (methyl mandelate) [(±)-**1**] was purchased from Sigma. 2-*O*- butanoyl-2-phenylacetic acid [(±)-**2**] was synthesized as previously described [24]. Other reagents and solvents used were of analytical or HPLC grade.

2.2. Standard enzymatic activity assay determination

This assay was performed by measuring the increase in absorbance at 348 nm ($\epsilon = 5.150\,M^{-1}\,cm^{-1}$) produced by the release of *p*-nitrophenol (pNP) in the hydrolysis of 0.5 mM pNPB in 25 mM sodium phosphate buffer at pH 7 and 25 °C. To initialize the reaction, 0.05–0.2 mL of lipase solution or suspension was added to 2.5 mL of substrate solution. One international unit of pNPB activity was defined as the amount of enzyme that is necessary to hydrolyze 1 μmol of pNPB per minute (IU) under the conditions described above.

When the enzyme was in presence of triton X-100 (a strong inhibitor), the enzymatic activity was assayed adding 0.001% of

CTAB at pH 7 and 25 °C, to revert that inhibition and to be able to detect some activity.

2.3. Immobilization of lipases on different supports

Different immobilized preparations were prepared following the procedures previously described: (i) ionically adsorbed lipase on solid supports coated with PEI [23] at pH 7 and 25 °C, (ii) covalent immobilization on CNBr-activated support at pH 7 and 25 °C [25], (iii) interfacial adsorption on a hydrophobic support, octyl-agarose at 5 mM sodium phosphate buffer at pH 7 [10].

Enzyme load was 1 mg/mL of support (that is approx. 1–2% of the maximum load) to prevent diffusion problems. Protein concentration was determined by the Bradford method [26].

2.4. Enzymatic hydrolysis of chiral esters

The activities of different immobilized preparations of Lecitase® Ultra were analyzed in the hydrolysis of different chiral esters. Substrate [(±)-**1**] was dissolved in a 3 mL solution of 10 mM sodium phosphate buffer to 5 mM of compound concentration at 25 °C at different pH conditions and 0.3 g of immobilized preparation were added. Substrate [(±)-**2**] was dissolved in a 1.5 mL solution of 10 mM sodium phosphate buffer to 0.5 mM of compound concentration at different pH conditions and 0.5 g of immobilized preparation were added. During the reaction, the pH value was maintained constant using a pH-stat Mettler Toledo DL50 graphic and the enzymatic activity was defined as micromoles of substrate hydrolyzed per minute per mg of immobilized protein. The degree of hydrolysis was analyzed by reverse-phase HPLC (Spectra Physic SP 100 coupled with an UV detector Spectra Physic SP 8450). For these assays a Kromasil C18 (25 cm × 0.4 cm) column was used, mobile phase acetonitrile–10 mM ammonium phosphate buffer at pH 2.95 (35:65, v/v) for compound **2**, (30:70, v/v) for compound **1** and UV detection was performed at 254 nm.

2.5. Determination of enantiomeric excess and enantioselectivity

The enantiomeric excesses (ee) of the released acid (at conversions between 10 and 15%) were analyzed by chiral reverse phase HPLC [21]. The column was a Chiracel OD-R, the mobile phase was an isocratic mixture of acetonitrile and $NaClO_4/HClO_4$ 0.5 M, (5:95, v/v) at a flow of 0.5 mL/min and UV detection was performed at 225 nm.

The enantiomeric ratio (E) was calculated using the equation reported by Chen et al. [27].

2.6. Stability of the different lipase immobilized preparations

The different immobilized preparations were incubated at the conditions described (pH, T, presence of organic co-solvents) and samples of these inactivating reactions were periodically withdrawn and their activity assayed as described previously.

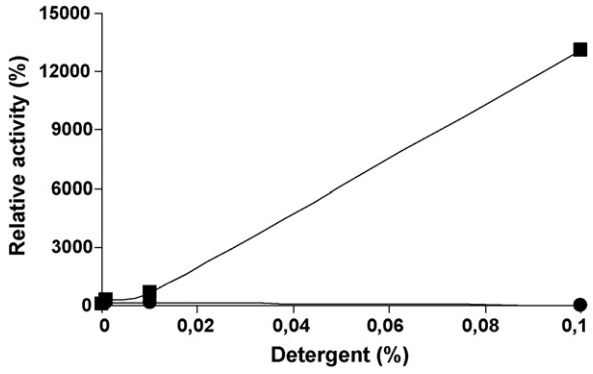

Fig. 1. Effect of detergent on the activity of Lecitase® Ultra against *p*-NPB. Experimental conditions are described in methods. The activity in absence of detergent is considered 100. Triton X-100 (circles), CTAB (squares).

3. Results and discussion

3.1. Influence of surfactants on the specific enzymatic activity

Lecitase® Ultra presents significant activity against *p*-nitrophenylbutyrate (*p*-NPB) (0.83 μmol/min/mg at 25 °C and pH 7, using a protein concentration of 8 ng/ml), but this activity becomes very increased in the presence of very small amounts of some detergents, like hexadecyltrimethylammonium bromide (CTAB) (Fig. 1). In fact, the use of 0.1% of this detergent permits to increase the enzyme activity by a 150-fold factor (the highest value we have found in literature of enzyme hyperactivation by adding detergents in aqueous media [12]).

However, other detergents, like Triton X 100, only permitted a small hyperactivation (1.5) when using very low concentrations (0.001%) while at 0.1%, produced a significant inhibition. Incubation of the enzyme at 25 °C and pH 7 in the presence of both detergents did not alter the initial values of activity, showing that Lecitase® Ultra is fully stable in the presence of these detergents. This suggests that the effect of the Triton X-100 is a direct inhibition but not an inactivation of the enzyme.

3.2. Immobilization of Lecitase® Ultra on octyl agarose and other supports

Around 80% Lecitase® Ultra esterase activity becomes adsorbed on octyl-agarose in 5 h at 5 mM of sodium phosphate. This enzyme increased the activity by a 13-fold factor during immobilization, as most of the conventional lipases [10], whose open form became adsorbed at low ionic strength at hydrophobic support by interactions between the hydrophobic areas around their active center, although the hyperactivation is on the top of the previously described lipase hyperactivations [10]. The enzyme could be desorbed from this hydrophobic support by addition of detergents (Triton X-100, CTAB). For example, the enzyme becomes fully desorbed from the support using 0.8% CTAB.

The desorbed enzyme (in the presence of the detergent) was immobilized following two different strategies. First, the ionic exchange on a support coated with PEI was intended [23]. While

Table 1
Residual activity of different Lecitase® Ultra immobilized preparations in the hydrolysis of *p*-NPB at pH 7 and 25 °C

Support	Immobilization yield	Residual activity[a] (%)	Residual activity[b] (%)
Octyl	100	–	1300
PEI	100	100	180
CNBr	100	nd	30

[a] Measured in the presence of the detergent.
[b] Measured after eliminating the detergent.

the enzyme desorbed from octyl agarose with Triton X-100 can be fully immobilized on the support, when the enzyme was desorbed using CTAB the immobilization was negligible. This effect may be associated to the cationic nature of CTAB, if the enzyme was loaded with molecules of this detergent (therefore, with a positive charge), it will not have a tendency to become adsorbed on an anionic exchanger support. The adsorption of the enzyme on the PEI coated support on the presence of Triton did not present a significant effect on its activity when assayed in the presence of the detergent (Table 1), suggesting that the immobilization did not have any negative effect on enzyme activity. After eliminating the detergent by exhaustive washing, the recovered activity was around 80% higher than the expected one. This increment in the enzyme activity could be explained by the fact that the enzyme was immobilized on the support in the presence of detergent, therefore as a monomer (dimers of the soluble lipases could yield less active forms than the monomer) [12]. Moreover, PEI could interact with the open form of the enzyme induced by the detergent, keeping (at less partially) the open structure induced by the detergent even when this has been eliminated [28] (Table 1). The second strategy was the covalent immobilization on CNBr-agarose. In this case, desorbed Lecitase® Ultra can be similarly immobilized using both detergents. In both cases, the enzyme activity was decreased significantly during immobilization (Table 1) activity recovery was 30%, measured in the absence of detergents.

3.3. Some features of the immobilized Lecitase® Ultra preparations

The effect of the detergents on the immobilized and fully dispersed enzyme molecules, should be attributed to the direct effect of the detergent on the individual enzyme molecules properties, in this case the breaking of likely lipase-lipase dimers is not possible [20]. CNBr-agarose immobilized enzyme is still strongly hyperactivated by the addition of small concentrations of CTAB (near to 60-folds using 0.01%), whilst higher concentrations produced a certain decrease in the observed hyperactivation (in opposition to what was observed using the free enzyme). The enzyme immobilized on PEI coated support presented a smaller but still very significant increment in its activity. The lower hyperactivation induced by the detergent could be due to the decreased mobility of the enzyme produced by the interaction with the polymer (in fact, this immobilization technique was used to keep altered structures of lipase B from *C. antarctica* under different conditions) [28], and that the enzyme presented already a certain increase in the activity after immobilization, perhaps by exhibiting a partially open form of the enzyme.

All immobilized preparations kept more than 90% of activity in a pH range from 5 to 8 for 1 week at 25 °C. At 45 °C and pH 7, the only immobilized preparation that remained fully active after 24 h was that obtained by adsorption on PEI-coated agarose. Curiously, the enzyme immobilized on octyl-agarose was the less stable biocatalyst retaining around 55% of the initial activity (Fig. 2A). Similar results were obtained at pH 5 (Fig. 2B), although in this case the octyl-agarose preparation lost almost 80% of the activity after only 24 h.

All the immobilized enzyme preparations showed a high stability in the presence of 30% (v/v) of organic cosolvents, like dimethylformamide, dioxane or acetonitrile at 25 °C. The enzyme immobilized in PEI-coated supports kept unaltered its activity after 24 h of incubation. The enzyme immobilized on octyl agarose suffered some inactivation only using dioxane (only by a 30% in 24 h) (results not shown).

These results suggest that all the Lecitase® Ultra immobilized preparations are quite stable in a broad range of conditions. However, the lower stability of the enzyme immobilized on octyl-agarose support when it is compared to the other immobilized preparations showed an opposite profile compared with the behavior of most standard lipases [29].

3.4. Resolution of (±)-1 and (±)-2 by different immobilized preparations of Lecitase® Ultra

Using (±)-**1**, the activity of all preparations was higher at pH 7 than at pH 5 (Table 2). However, the most active preparation

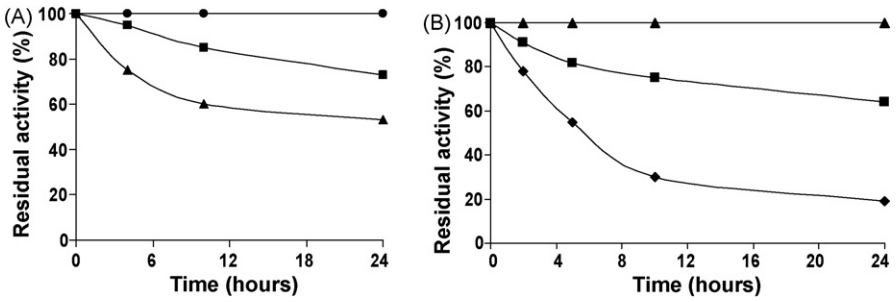

Fig. 2. (A) Thermostability of the different preparations of Lecitase® Ultra. Inactivation was carried out in 25 mM sodium phosphate buffer, pH 7 and 45 °C. CNBr-lecitase (square), octyl-lecitase (triangles), PEI-lecitase (circles). (B) Thermostability of the different preparations of Lecitase® Ultra. Inactivations were carried out in 25 mM sodium phosphate buffer, pH 5 and 45 °C. CNBr-lecitase (squares), PEI-lecitase (triangles); Octyl-lecitase (rhombus).

Table 2
Hydrolytic resolution of [(±)-**1**] catalyzed by immobilized Lecitase® Ultra preparations at different pH values

Support	pH	Specific activity	*E*	Isomer
Octyl	5	0.026	3.3	R
	7	0.12	3.0	R
PEI	5	0.047	7.5	R
	7	0.083	3.3	R
CNBr	5	0.0014	2.2	R
	7	0.0022	5.8	R

Experiments were performed at 25 °C. Specific activity is in μm/min/mg of enzyme. *E* was calculated using enantiomeric excess of product at 10–15% conversion.

at pH 7 was the Lecitase® Ultra immobilized on octyl agarose, while at pH 5 the most active one was that immobilized on PEI-coated supports, being the CNBr preparation the less active preparation in all cases.

Using [(±)-**2**]**,** the most active preparation was, at both pH values, the octyl-agarose one. The enzymatic activity experimented a decrease when the reaction was performed at pH 5 (conditions where the carboxylic moiety presents a lower ionization) for all preparations (Scheme 1). This effect was especially remarkable in the PEI one where the activity decreased up to 160 times (Table 3).

These data already reflect that the immobilized enzymes have a very different sensitivity to the experimental conditions.

The enantioselectivity of the immobilized Lecitase® Ultra was not very high against (±)-**1** (Table 2), always favoring the hydrolysis of the R isomer (Scheme 1). However, it depended on the enzyme preparation. The highest enantiomeric ratio was obtained using the Lecitase® Ultra adsorbed on PEI-agarose at pH 5 ($E = 7.5$). The CNBr preparation also gave a moderate enantioselectivity, with the highest value observed at pH 7 ($E = 6$) (Table 2).

The effect of the pH on the *E* value was very different depending on the different preparations. The observed *E* value using the enzyme immobilized on octyl-agarose was almost unaltered by the change in the pH value, whereas an increase in enantiomeric ratio was observed to the PEI-preparation after decreasing the pH from 7 to 5. The CNBr immobilized enzyme presented an opposite behavior with a maximum in *E* value at pH 7.

Table 3
Hydrolytic resolution of [(±)-**2**] catalyzed by immobilized Lecitase® Ultra preparations at different pH values

Support	pH	Specific activity ($\times 10^{-3}$)	*E*	Isomer
Octyl	5	3.2	>100	R
	7	0.043	12	R
PEI	5	1.6	8	R
	7	0.01	2	R
CNBr	5	0.71	26	S
	7	0.033	1.3	S

Experiments were performed at 25 °C. *E* was calculated using enantiomeric excess of product at 10–15% conversion.

Using (±)-**2**, results were even more different depending on the immobilized preparation (Table 3). The enzyme immobilized on octyl-agarose presented the highest *E* value ($E > 100$) when the reaction was performed at pH 5 (Table 3), presenting an ee > 99%. Thus, it was possible to obtain the pure R-**3** at 50% conversion (Scheme 1). The *E* value was much lower when the reaction was carried out at pH 7 ($E = 12$). The Lecitase® Ultra immobilized on PEI-coated support gave lower E values at both pH values. Finally, the CNBr immobilized preparation exhibited an inversion in the estereoselectivity, hydrolyzing the S isomer more rapidly, reaching an E value of 26 when the reaction was performed at pH 5 (Table 3).

Thus, Lecitase® Ultra seems to be extremely sensible to the immobilization protocol, even more than conventional lipases [21].

4. Conclusions

Lecitase® Ultra preparation exhibits many properties similar to conventional lipases: it is hyper-activated by some detergents [12], it becomes adsorbed on octyl-agarose at low ionic strength exhibiting a certain hyperactivation during this process [10], and is able to recognize very different esters, like (±)-**1** and (±)-**2**, exhibiting very significant E values in certain cases. Lecitase® Ultra may be strongly modulated by immobilization and medium engineering as most of the standard lipases [21]. Moreover, using (±)-**2**, as substrate, the octyl and CNBr preparations at pH 5 gave an inverse enantiopreference. In fact, this inversion in enantiopreference when using different immobilization protocols suggests that Lecitase® Ultra may be among the enzymes more sensitive to this strategy.

Therefore, depending on the enzyme preparation used when the reaction was performed at pH 5, enantiomerically pure R-**3** was obtained at 50% conversion using the octyl-lecitase preparation as biocatalyst, whereas R-**2** was got quite pure at 56% conversion using CNBr-lecitase preparation as biocatalyst (ee > 95%).

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