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Alteration of the activity and selectivity of immobilized lipases by the effect of the amount of water in the organic medium

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Abstract

Lipase from *Candida cylindracea* has been covalently immobilized on agarose, silica and alumina. These enzymatic derivatives are inactive in synthesis after lyophilization (48 h) if an aqueous buffer is not added to the organic reaction medium. The addition of water restores the enzymatic activity of the immobilized lipase on agarose, but not on hydrophobic supports such as silica or alumina. Unlyophilized derivative on silica is more stable and active than the native enzyme in its optimum hydration conditions. In the esterification of racemic ibuprofen in isooctane, native and immobilized lipases are selective towards the *S*(+) enantiomer. Immobilized lipase on silica and native lipase have the same synthetic activity and selectivity, but the immobilized derivative is four times more stable at 25°C than its soluble counterpart.

Keywords: *Candida cylindracea*; Enantioselectivity; Esterification; Ibuprofen; Immobilized enzymes; Lipases

1. Introduction

The interest of lipases (EC 3.1.1.3) is directed at their widespread application in the resolution of racemic mixtures through esterification reactions in organic solvents with low amount of water. This reaction is often more enantioselective than the hydrolysis of the corresponding ester [1,2].

Among all lipases, the well-known lipase from *Candida cylindracea* has been used in esterification and transesterification reactions in organic media, with acids and alcohols with different structures [3–10]. In these synthetic processes, this biocatalyst has been employed in its native form [11–14] as well as adsorbed [15,16], gel-entrapped [17], in reverse micelles [18,19] and covalently bonded to a support [20]. The immobilization of lipase can offer several advantages such as re-utilization, improvement of the enzymatic stability, etc. Moreover, the immobilization process can even enhance the synthetic activity of the lipase relative to the native form; for instance Gray et al. [20] achieved more than 100% of activity in the synthesis of menthol esters by transesterification using immobilized derivatives of lipase from *Candida cylindracea*. These biocatalysts were prepared by adsorption on polyethylene, and by covalent immobilization to cellulose (both supports activated with glutaraldehyde).

As an interesting application for obtaining the *S*(+) enantiomer of (±) 2-aryl propionic acids, which has the desired antiinflammatory effect, lipase from *Candida cylindracea* has been used in the resolution of (±) 2-(4-isobutylphenyl) pro-

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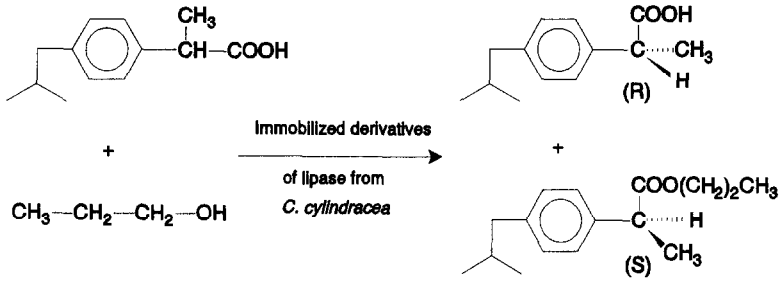


Scheme 1.

pionic acid (ibuprofen) by ester synthesis in organic media. To provide some instances of this approach, Mustranta [21] reported high ester yields and complete resolution of ibuprofen using lipase from *Candida cylindracea* in native form in the presence of very hydrophobic organic solvents (with high log*P*). Hedström et al. [22] achieved the resolution of this antiinflammatory drug in the same Mustranta's conditions but using a water-in-oil microemulsion (AOT/isooctane), in which the lipase displayed higher enantioselectivity and lower reaction rate than in the pure organic solvent system (isooctane).

In the resolution of racemic mixtures, the literature data are contradictory, since the amount of water in the reaction medium can increase [23,24] or decrease [25,26] the enantioselectivity of native lipases. In addition, as far as we know, little work has been carried out on the influence of water in resolution catalyzed by immobilized lipases.

In the present paper we analyze this topic with lipase from *C. cylindracea* immobilized on three different supports: agarose, alumina and silica. These supports were chosen due to their different mechanical and chemical properties, and their corresponding immobilized derivatives were employed in the resolution of (±) ibuprofen by enantioselective esterification in isooctane (Scheme 1).

2. Materials and methods

General. Lipase from *Candida cylindracea* (EC 3.1.1.3; type VII, containing 1010 U/mg solid, using olive oil as substrate) and 1-propanol were obtained from Sigma (St. Louis, MO, USA). Racemic ibuprofen was a gift from Boots Pharmaceuticals (Nottingham, UK). Isooctane (with analytical grade), alumina and silica were from Merck (Germany). Agarose gel beads (Biogel A-150, 100–200 mesh) were from BioRad Laboratories (Richmond, VA, USA).

General procedure for immobilization. The activation of agarose [27] and inorganic supports [28] was carried out according to the methodologies previously described by the authors. The immobilization of lipase was performed at 4°C during 3 h with low stirring. One gram of each support was added to different enzyme solutions: 250 mg (agarose) or 400 mg of commercial lipase (silica and alumina) in 10 ml of Tris/HCl 0.1 M (pH = 8.0) buffer. After the desired contact time, the insoluble derivative was filtered and washed with standard buffer. The percentage of immobilized enzyme was determined by the difference between the initial activity of the native enzyme and the activity of the filtrate. The wet derivatives were stored at 4°C or lyophilized during 48 h. Thermal stability of wet derivatives at 25°C was studied as described previously [28].

Enzyme assays. The hydrolysis of olive oil emulsion (lipase activity) was carried out in 1 mM sodium phosphate/NaOH buffer (pH = 7.5) at 35°C. The acid released was continuously titrated to pH = 7.5 with the aid of a pHstat Crison model MicroTT 2022. Several NaOH solutions (1 to 10 mM) were used as titrating agents. The stirrer speed was 50% and the total reactor volume was 5 ml.

The catalytic efficiency of immobilized derivatives, was determined – using initial reaction

rates – as the ratio between the specific enzymatic activities of 3 mg of native enzyme and the amount of immobilized derivative which contains 3 mg of enzyme. All of the data are the average of triplicate samples and are reproducible within ±5%.

General procedure for esterification. The reaction mixture was composed of isooctane (10 ml), racemic ibuprofen (66 mM) and 1-propanol (66 mM). The reaction was started by adding different amounts of the immobilized derivatives and the required amount of Tris/HCl 0.1 M (pH = 7.0) buffer to the solution. The reactions were performed at 30°C by stirring (500 rpm) in 25 ml flasks. Periodically, 100 μl of the solution were added to 1.4 ml of isooctane to be analyzed the ester conversion by capillary gas chromatography.

Gas chromatography analysis. GC was performed in a Shimadzu GC-14A gas chromatograph equipped with a FID detector, a split injector (1:2) and a SPB®-1 sulfur column 15 m × 0.32 mm (Supelco Inc. Bellafonte, PA, USA). Injector temperature was 300°C and the detector temperature was 350°C; oven temperature was maintained at 180°C. Carrier gas was nitrogen with a flow of 12 ml/min. An external standard method was employed to quantify the remaining acid and the formed ester.

Enantiomeric excess determination. The enantiomers of the unreacted ibuprofen were separated by HPLC using a chiral column (Chiralcel OD, Daicel Chemical Industries Ltd., Japan). The mobile phase was a mixture of n-hexane/isopropanol/trifluoracetic acid (100:1:0.1 v/v/v) at a flow rate of 1 ml/min. UV detection at 254 nm was used for quantification at the ambient temperature.

3. Results and discussion

Characterization of the immobilized derivatives. The results of the immobilization process of lipase from *Candida cylindracea* on tosylated agarose and inorganic supports (SiO_2 and Al_2O_3) activated via trichlorotriazine are shown in Table 1.

The percentages of immobilized lipase are similar to those reported by Lavayre and Baratti [29] using Spherosil as support. However, the load of enzyme is higher than the value of 0.5 mg of enz./ml gel of Sepharose 4B reported by Kilara et al. [30], Shaw et al. [31] (19 mg/ml gel of chitosan) or Carta et al. [32] (10 mg/ml gel using nylon as support). Therefore our methodologies are interesting for the immobilization of this enzyme. The milligrams of active lipase bound per gram of support is greater in the agarose than in the inorganic supports.

The immobilized derivative on agarose has the highest value of half-life at 25°C (190 h). Nevertheless, all the enzymatic derivatives are between 5 to 12 times more stable than native enzyme (Fig. 1).

Synthetic activity of the immobilized derivatives. Influence of the amount water. The amount of water present in the microenvironment of

Table 1
Characterization of immobilized derivatives of lipase from *Candida cylindracea*

Support	Lipase added (mg)/ g support	(E_{inm}) [d] (%)	mg of immobilized lipase/g support	Catalytic efficiency (%) [e]	mg of active lipase/ g support	Half-life (h) at 25°C [f]
Agarose [a]	250	38.9	97.4	76	74	190
SiO_2 [b]	400	22.7	90.9	44	40	80
Al_2O_3 [c]	400	36.6	146.0	26	38	75

[a] Activation degree = 8.8 μmol tosyl/g agarose.
[b] Activation degree = 1208.7 μmol of TCT/g silica.
[c] Activation degree = 1029.8 μmol of TCT/g alumina.
[d] Referred to the native lipase added in the immobilization process.
[e] Lipase activity using olive oil emulsion.
[f] The half-life of the native enzyme at 25°C is 20 h.

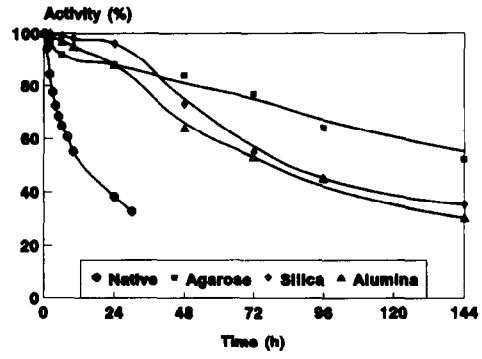


Fig. 1. Thermal stability of native and immobilized lipase from *Candida cylindracea* stored at 25°C.

immobilized enzymes on solid supports has been quantified by Mattiasson et al. [33] considering the aquaphilicity of the support (Aq). We have recently reported the change in the aquaphilicity induced by the lyophilization of α-chymotrypsin immobilized derivatives on tosylated Sepharose [34,35]. To this end, we have studied the influence of the lyophilization and the amount of aqueous buffer (0.1 M Tris/HCl [36]) on the catalytic activity of the immobilized derivatives (Table 2).

We have observed that all the enzymatic derivatives need water to be active. Lyophilization of the biocatalysts for 48 h practically removes the water shield of the enzyme, giving inactive enzymatic derivatives if an anhydrous organic solvent is used as the organic medium. This behaviour is not related to the nature of the support (entries 1, 6 and 10). This effect has been reported in the literature for this lipase in its native form [37].

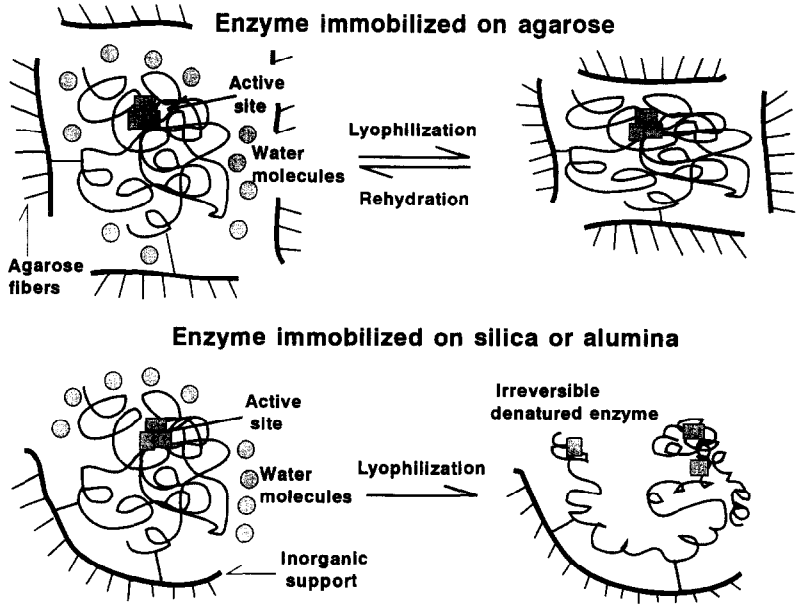
The addition of 500 μl of water to the medium (10 ml of isooctane) reactivates the lyophilized derivative on agarose. This result may be explained as a reversible rehydration of agarose (a highly hydrophilic support whose aquaphilicity is 13.8 [33]), which restores the water shield of the enzyme. A similar behaviour has previously been described by Martin et al. for α-chymotrypsin covalently bonded to agarose [34,35]. When the amount of added water is greater than 500 μl, the synthetic activity is not increased (entries 2 and 3) because these new water molecules are absorbed by the bulk of agarose (250 mg) instead of being located in the microenvironment of the enzyme.

From our results, we can conclude that this amount of water first added to the solution is quickly absorbed in the surface of the agarose, restoring the structure of this polymer and the water shield of the lipase (Scheme 2). Both effects produce a change from the rigid dehy-

Table 2
Influence of the lyophilization and influence of the presence of water on the esterification of racemic ibuprofen catalyzed by immobilized lipase from *Candida cylindracea* in isooctane

Entry	Immobilized derivative	mg derivative/10 ml solvent	lipase loading [a]	mg active lipase/10 ml of solvent	Tris/HCl buffer added (μl) [b]	Synthetic activity at 96 h [c]
1	Lyophilized agarose	250	296	74	0	0
2	Lyophilized agarose	250	296	74	500	0.015
3	Lyophilized agarose	250	296	74	1000	0.012
4	Unlyophilized agarose	1000	74	74	1000	0.012
5	Unlyophilized agarose	1000	74	74	2500	0.016
6	Lyophilized silica	1000	56	56	0	0
7	Lyophilized silica	2000	56	112	1000	0
8	Unlyophilized silica	2000	40	80	1000	0.031
9	Unlyophilized silica	2000	40	80	1500	0.014
10	Lyophilized alumina	1000	57	57	0	0
11	Lyophilized alumina	2000	57	114	1000	0
12	Unlyophilized alumina	2000	38	76	1000	0.009
13	Unlyophilized alumina	2000	38	76	1500	0.021

[a] mg of immobilized active lipase per gram of lyophilized or dry support.
[b] The amount of aqueous buffer is added to the organic solution.
[c] The synthetic activity is expressed in μmoles of esterified ibuprofen per mg of active lipase and hour. Conditions: 66 mM (±) ibuprofen + 66 mM 1-propanol in 10 ml of isooctane; $T = 30°C$; stirring speed = 500 rpm.



Scheme 2.

drated molecule of enzyme to its active and flexible conformation (entry 2 versus 1). Further additions of water do not alter the microenvironment of the enzyme but rehydrates the agarose, so enzymatic activity does not change and the same reaction yield is achieved (entries 2 and 3).

In contrast to agarose, the lyophilization of the derivatives on inorganic supports such as alumina and silica (aquaphilicity values of less than 1) produces a severe dehydration of the immobilized enzyme whose structure is strongly distorted, due to the rigid nature of these solids and the flexibility of the enzyme. Therefore the distortion of the enzyme followed by the loss of activity is produced by the removal of the water molecules from the microenvironment of the lipase (Scheme 2). The initial and active conformation is not restored although additional amounts of water are added to the medium (entries 6 vs. 7 and 10 vs. 11). The reversibility in the dehydration of agarose derivative (entries from 2 to 5) is not observed in the lyophilized inorganic derivatives of lipase, so we have worked with the unlyophilized derivatives which are active in the organic medium.

When we compare the synthetic activity of the different derivatives which have approximately the same amount of immobilized lipase under the same hydration conditions (entries 4, 8 and 12), we can observe that the enzymatic derivative immobilized on silica is more active than on agarose, and the least active is the derivative prepared on alumina. This derivative on alumina increases its activity with additional water (entry 13) whereas agarose maintains its activity, even with higher amounts of aqueous buffer (entry 5) and the silica derivative diminishes its synthetic activity when we increase the amount of water (entry 9). In this last case, the difference in the enzymatic activities caused by a further addition of water between the enzymatic derivatives on inorganic supports, may be related to a high hydration of the microenvironment of the lipase immobilized on silica, which increases hydrolase activity. In these conditions *S*-acid is esterified and the formed *S*-ester quickly hydrolyzed, giving low reaction yields.

So we can conclude that lipase immobilized on hydrophobic supports gives greater reaction yields than lipase immobilized on agarose in their optimum conditions. These results agree with those reported in the literature [31], which showed higher synthetic activities for immobilized lipases on hydrophobic than on hydrophylic polymers.

Table 3
Influence of water on the enantioselectivity of native and immobilized lipase from *Candida cylindracea* in the esterification of racemic ibuprofen with n-propanol

Entry	Derivative [a]	Tris/HCl buffer added (μl)/10 ml of isooctane	c (%) [b]	t (h)	ee (%) [c]
1	Native	200	12	192	15.7
2	Native	1000	40	192	71.7
3	Lyophilized agarose	500	25	144	22.0
4	Lyophilized agarose	1000	17	144	27.0
5	Unlyophilized agarose	1000	20	168	35.4
6	Unlyophilized agarose	2500	29	168	37.2
7	Unlyophilized silica	1000	40	192	75.3
8	Unlyophilized silica	1500	21	192	24.6
9	Unlyophilized alumina	1000	23	192	16.9
10	Unlyophilized alumina	1500	32	192	16.0

[a] The amount of active enzyme (native or immobilized lipase) added to 10 ml of isooctane was 75 mg in all the experiments. Conditions: 66 mM (±) ibuprofen +66 mM 1-propanol; $T^a = 30°C$; 500 rpm.
[b] Ester yield.
[c] Enantiomeric excess of remaining $R(-)$ ibuprofen.

Resolution of (±) *ibuprofen.* The effect of the nature of the support on the lipase stereoselectivity is shown in Table 3. In all cases, the immobilized and native enzyme esterifies the $S(+)$ enantiomer. Therefore the immobilization process described in this paper does not alter the stereoselectivity of the biocatalyst. The enantiomeric excess (ee) has been calculated relative to the unreacted acid which was always $[R(-)] > [S(+)]$. The enantioselectivity observed corresponds to that reported for the native lipase by Mustranta [21] and by Hedström et al. [22].

We can observe different behaviour between the native and the immobilized lipase. The enantiomeric excess obtained with the native enzyme increases with the amount of water (buffer added to the medium) as described by Högberg et al. [24], whereas the immobilized derivatives are not affected by this additional amount of water, except in the case of the derivatives on silica. In this last case, it is interesting to note how an excess of water in the microenvironment of the enzyme increases the hydrolase activity towards the formed *S*-ester. As a consequence the remaining acid becomes more racemic (the ee diminishes).

The influence of the support on the enantioselectivity is also compared in Table 3 (entries 2, 4, 5, 7 and 9). At the same final reaction time (192 h) and at the same amount of water (1 ml of buffer), only the immobilized lipase on silica displayed the same enzymatic activity and enantioslectivity as the native form. Therefore, we can conclude that this silica derivative, owing to his high thermostability (Fig. 1), is a step forward in the field of immobilized lipases as an improved biocatalyst in the enantioselective resolution of racemic ibuprofen in organic media.

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