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Kinetic and spectroscopic behaviour of a lipase–microgel derivative in aqueous and micellar media

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Abstract

The kinetic and spectroscopic behaviour of the native and immobilized isolipase B from *C. rugosa* were compared in water and in micelles. A negative interaction of the lipase with the microgel matrix reduced the catalytic efficiency by 34 times and produced a blue shift in the protein emission spectrum in water. However, the immobilization caused the opposite variation in its kinetic and spectrofluorimetric behaviour in the micellar medium. The crosslinked polymer (hydrodynamic radius 400 ± 20 Å) modified the micellar aggregate. It increased the water activity of poorly hydrated micelles (small $W_0 = [H_2O]/[\text{surfactant}]$ values). The emission fluorescence of 2-naphtol, solubilized through the tensioactive molecules in the micellar interface, varied according to the increase of the micellar size. This microgel increased the polarity of the micellar aqueous phase, as shown in the red shifted spectrum of 1-naphthol-6,8-disulphonate. The protein was sensitive to this change of polarity and its fluorescence emission was also red shifted in the presence of the microgel. These changes on the micellar droplets and the subsequent modification on the Trp environment of the immobilized protein affected the k_{cat} and $K_{m,app}$ values. The polymer decreased the stability of lipase B in water. This negative enzyme–microgel interaction was favoured in the micelles of the smallest W_0 values. A model to explain the activity and stability of the enzyme–microgel system in micelles has been proposed here. This model considers the structural variations in the micelle because of the microgel (A_w, droplet size). © 1998 Elsevier Science B.V.

Keywords: Lipases; *Candida rugosa*; Microgel; Reverse micelles; AOT; Fluorescence

1. Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are enzymes which in vivo hydrolyze the long chain aliphatic esters of triglycerides [1]. Lipase activity is greatly increased at the lipid–water interface [2]. This interfacial activation is associated with conformational changes which includes a large displacement of a loop that covers the active site (lid) and stabilizes the interaction with the substrate [3–8].

In the case of *C. rugosa* the isolipases present kinetic phenomena of interfacial activation and the 'open' and 'closed' conformations of the main isoform have been crystallized [9–11]. The isolation of different isoenzymes from *Can-*

Abbreviations: AOT, Sodium bis-(2-ethylhexyl) sulphosuccinate; pNPB, *p*-nitrophenyl butyrate; NOH, 2-naphthol; NDSOH, 1-naphthol-6,8-disulphonate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride; W_0, $[H_2O]/[\text{surfactant}]$; k_2^0, $k_{cat}/K_{m,app}$; $t_{50\%}$, incubation time at which the biocatalyst lost 50% of its initial activity.

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dida rugosa or *cylindracea* lipase has been previously reported [12–14]. The isolipase B from this microorganism obtained in our laboratory has a molecular mass of 60 kDa [14,15].

Reverse micellar systems are formed by nanometer-sized water droplets dispersed in organic media by the action of surfactants [16]. These nanoaggregates have been used to work with different types of enzymes in similar conditions to those in vivo [17,18]. The enzyme–micelle interactions are relevant for the activities of hydrolytic enzymes [19–21]. Besides the static view of the crystal structures, reverse micelles are apt systems to study the structure–activity relationship of lipases in situ in the presence of interfaces. In the case of empty AOT (sodium bis-(2-ethylhexyl) sulphosuccinate) micelles, their micellar size increases when $W_0 = [H_2O]/[AOT]$ enhances.

The micellar aggregates should be appropriate systems for enzymes working in interfaces, due to their high interfacial area. But the unfavourable partition of the apolar substrates between the interface and the continuous organic phase may partly explain the inhibition effect of reverse micelles [22]. The lipase hydrophobicity (related to the degree of enzyme penetration at the interface) has been found relevant to mitigate the inhibition effect of charged micelles [22,23]. Enzymes may be covalently bound to colloidal cross-linked polymer particles [24], obtained by emulsion polymerization (microgels) [25], whose diameters vary between 40 and 300 nm. These microgel–enzyme derivatives may be dissolved in water, in organic media as colloidal sols in the presence of a small amount of water (ca. 4%) [26], or into the aqueous nanodroplets of a micellar solution. The polymer has an apolar inner with the polar groups oriented to its surface in aqueous media [24,25].

In this paper we comparatively studied the kinetic and fluorimetric behaviour of a native lipase and a lipase–microgel derivative in two media, water and AOT micelles. The effect of the microgel on the lipase activity and stability was studied in these two systems. In micelles, the enzyme–microgel derivative modified the droplet size, due to the big microgel volume. Information about the relevance of the micellar size at constant hydration degree (W_0) was also obtained with this system. Isolipase B from *Candida rugosa* was chosen for this study.

2. Materials

Lipase from *Candida cylindracea* (CCL, type VII), sodium bis-(2-ethylhexyl) sulphosuccinate (AOT), *p*-nitrophenyl butyrate (pNPB) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) were obtained from Sigma. 2-Naphthol (NOH) was from Aldrich and 1-naphthol-6,8-disulphonate (NDSOH) from Fluka. Acrylamide and N,N,N',N'-tetramethylethylenediamine were from Merck and N,N-methylene-bisacrylamide from Serva. Other monomers purchased from Aldrich were freed from inhibitors before use by either distillation in vacuo or by swirling with dilute NaOH, separating the organic layer and drying, where appropriate, with $MgSO_4$.

3. Methods

3.1. Purification of lipase B from Candida cylindracea

Purification was carried out as previously described [14].

3.2. Microgel production

Microgels possessing carboxylic acid were prepared as described elsewhere [27]. The monomer feed compositions for the polymer synthesis was: methyl methacrylate, 45%; 2-ethoxyethyl methacrylate, 30%; ethyleneglycol dimethacrylate, 10% and acrylic acid, 15 mol%.

3.3. Conjugation of enzyme with polymer

Enzyme (9.3 mg) in water (1.9 ml) was mixed with the aqueous solution of polymer (1.9 ml) and the solution adjusted to the indicated pH. The mixture was kept for the indicated time with EDC (36 mg). The enzyme–polymer conjugate was then separated from the native enzyme and the urea by-product in a molecular exclusion column, Sephacryl S-200.

The total protein was assayed by the Peterson method [28]. At the microgel concentrations used, any interference of it in the Peterson method was detected. The activities of native and immobilized lipases were spectrophotometrically assayed with pNPB.

3.4. Enzyme solutions in AOT systems

The microemulsions contained AOT 0.1 M and the corresponding buffer (acetate buffer, pH 4.5–5.0; or 2-(*N*-morpholino)-ethanesulphonic acid (MES), pH 5.5–6.5; or 3-(*N*-morpholino)-2-hydroxypropanesulphonic acid (MOPSO), pH 7.0; or *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulphonic acid (HEPES), pH 7.5; or *N*-(2-hydroxyethyl)piperazine-*N*′-3-propane sulphonic acid (EPPS), pH 8.0). *n*-Heptane dried with molecular sieves (0.0005% w/w of water by Karl Fischer) was used as the continuous phase of micellar solutions.

3.5. Enzyme stability study

The stabilities of the native and immobilized lipases in water and in micellar media ($W_0 = 5$, 12.5 and 30) were studied at their respective optimal pHs for their hydrolytic activities in these systems. Other pH values were also studied in the case of the native lipase. Samples of 25 ml containing 0.042 mg protein/ml of the native or modified lipase were incubated in micellar systems at 200 rpm and 30°C. The incubations in aqueous media contained 0.16 mg/ml of the native or conjugated lipase. After different times an aliquot of the micellar (or aqueous) solution was taken to measure the remaining enzymatic activity using pNPB hydrolysis in water as the standard reaction. $t_{50\%}$ was defined as the incubation time at which the biocatalyst has 50% of its initial activity.

3.6. Determination of the enzymatic hydrolytic activity

The hydrolytic activities of these two lipases were measured by following the accumulation of *p*-nitrophenol in a Kontron 930 UVIKON spectrophotometer equipped with thermostated cells at 30 ± 0.1°C. The monitoring wavelength was selected at the isosbestic point of the nitrophenol/nitrophenolate couple; 346 nm in the assays in water (molar extinction coefficient: 4800 M^{-1} cm^{-1}, [29]). The isosbestic points in AOT systems have been previously reported in Ref. [23]. When necessary, small corrections were made for the non-enzymatic-catalyzed rates of hydrolysis. The initial rate was obtained in less than 2 min. Thus, a possible influence of enzyme stability was avoided.

Michaelis–Menten parameters (K_m and k_{cat}) were obtained by curve fitting and statistical treatment of the data, performed using the RFFIT interactive programme of the SIMFIT package [30]. This programme performed regressions of the Michaelis–Menten equation and afforded the parameter values and their statistical confidence limits. From these values, the specificity constant, k_2^0 ($k_2^0 = k_{cat}/K_m$) was calculated. Concentration units for the enzyme and reactants were referred to the total volume of the micellar system.

3.7. Fluorescence studies of lipases

Fluorescence spectra of the purified isolipase B (native and immobilized) in 0.1 M buffer, were compared with their spectra in the micellar system of AOT ($W_0 = 5$, [buffer] = 1.5 mM), at the corresponding optimal pH of the enzymes in each medium. Protein concentration in the cuvette was 0.024 mg/ml in buffer and 0.011

mg/ml in the micellar solutions. Spectra were recorded at 30 ± 0.1°C at 295 nm excitation and 305–450 nm emission, in a Perking Elmer fluorescence spectrometer LS 50B. Emission and excitation slit widths were 2.5 mm for the spectra in water and 5 mm for the AOT systems. Spectra were uncorrected for the instrument sensitivity, but Raman emission of the solvent (buffer or micellar solution) was subtracted. Spectra were also corrected by the inner filter effect, which was determined according to the equation [31]

$$F_c = F_m 10^{(A_{ex}+A_{em})/2},$$

where F_c is the corrected fluorescence intensity, F_m is the measured fluorescence intensity after correction for Raman emission of the solvent and A_{ex} and A_{em} are the absorbances measured at the excitation and emission wavelengths. In all experiments A_{ex} was always less than 0.1.

3.8. Fluorescence studies of probes solubilized in the micelles

The fluorescence emission of NOH and NDSOH probes in AOT micelles ($W_0 = 5$, pH = 5) were carried out at 30 ± 0.1°C, in the absence and presence of microgel. The spectra of NOH were recorded at 315 nm excitation and 322–550 nm emission and in the case of NDSOH at 310 nm excitation and 350–600 nm emission. In both cases the slit widths were 2.5 mm. Raman emission of the micellar solution and the microgel was subtracted. Spectra were also corrected by the inner filter effect as above. In all experiments A_{ex} was always less than 0.1.

3.9. Determination of the water activity

The water activities (A_w) of the micellar systems were determined with a Thermoconstanter Novasina RTD-200 TH-2 apparatus at 30 ± 0.1°C. A sample of 5 ml, containing or not the enzyme or the acrylic support, was used, corresponding to the sample volume used in the activity studies.

4. Results

4.1. Hydrolytic activities of native and immobilized lipases in water and reverse micellar systems

4.1.1. Optimal pHs of lipases

Fig. 1 shows the variation of initial rate of the pNPB hydrolysis in water, catalyzed by the native and immobilized lipases versus pH at 30 ± 0.1°C. The enzyme linkage to the microgel decreased the optimal pH in water from 7.5 to 6.5. This showed the unfavourable enzyme–microgel interactions at high pHs, due to the effect of dissociated polar groups at the microgel surface.

In Fig. 2A and B the variations of the initial rate of the native and conjugated lipases are presented, respectively, with the pH of the buffer used to prepare the micellar systems ($W_0 = 3$–30). The negative charge of the micellar interface also decreased the optimal p values of the native and immobilized lipases. The reductions

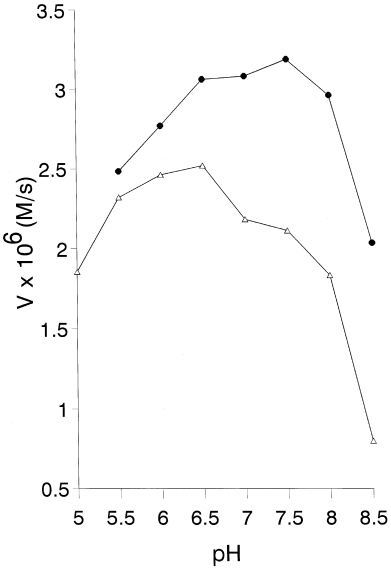

Fig. 1. Variation of the initial rate of pNPB hydrolysis catalyzed by the native (●) and immobilized (Δ) lipases with pH in aqueous medium. Conditions: native lipase 13.9 nM, lipase-microgel 26.3 nM, pNPB 0.558 mM, buffer 0.1 M and 30 ± 0.1°C.

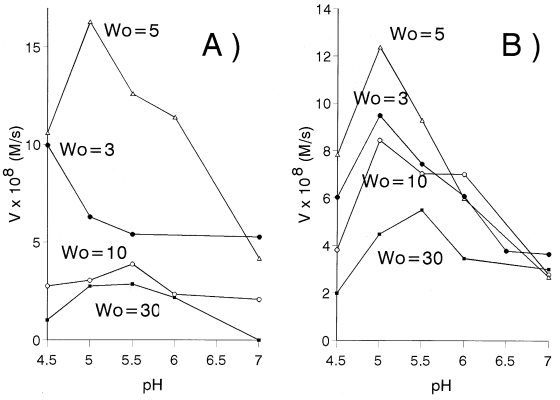

Fig. 2. Dependence of the activities of lipases on the pH in the AOT systems at different W_0 values. Conditions: [pNPB] = 2.36 mM; [buffer] = 2.2 mM and 30 ± 0.1°C. (A) native enzyme 8.33 nM; (B) lipase–microgel 7.9 nM.

of the optimal p values were more important in the micelles of lower W_0 values. Thus, the optimal pH of native lipase B decreased from 7.5 in water to pH 5.5 when $W_0 = 10–30$, to pH 5.0 in micelles of $W_0 = 5$, and to pH ≤ 4.5 at $W_0 = 3$. The immobilized enzyme was slightly less sensitive to this effect, and the optimal p values were the same at $W_0 = 3–10$ (pH = 5). Consequently, the minimum optimal pH of the lipase–microgel in micelles was not as low as in the case of the native enzyme. In Fig. 2A, the different reaction profile in micelles of $W_0 = 3$ with respect to that found in micelles of higher values of W_0 is apparent and it corresponds to a shift of the optimal pH at a value lower than the lower limit of the pH range herein studied.

The microgel surface is anionic due to the presence of acrylic groups in its composition. The tensioactive used here is also anionic and considering the variation of pH_{opt} of lipase–microgel with W_0, the decay of pH_{opt} of the immobilized lipase in micelles (Fig. 2) was not only due to the enzyme–microgel interaction but also to the anionic micellar surface. The two interactions, enzyme–microgel and enzyme–micelle, decreased the optimal pH of this lipase.

4.1.2. The kinetic constants

The kinetic parameters of the lipase B and lipase B–microgel were determined in water and in AOT micelles ($W_0 = 5$), at their respective optimal pH's. The values found are given in Table 1. In micelles, the substrate partition between the organic continuous phase and the interface, where the reaction takes place was not considered. This work was not focussed on the substrate partition and its effect on the kinetics. The comparative study of the native and immobilized lipases in micelles was carried out considering that the substrate partition should not significantly vary at a given surfactant concentration ([AOT] = 0.1 M) and $W_0 = 5$. In consequence, the apparent values of K_m ($K_{m,app}$) were calculated and compared.

The specificity constant, $k_2^0 = k_{cat}/K_m$, of this isolipase in water decreased by 3.4 times after its linkage to the microgel, due to the negative effects of the microgel linkage on the lipase affinity for the substrate (K_m) and in the catalytic constant. The possible conformational change of the protein were considered (see the fluorescence studies below). However, this type of enzyme–microgel conjugation gave rise to a modest enhancement of k_2^0 in the AOT system.

Table 1
Kinetic parameters of the native and immobilized lipase B for the hydrolysis of pNPB in buffer and AOT micelles

Enzyme	System	pH_{opt}	k_{cat} (s^{-1})	$K_{m,app}$ (mM)	k_2^0 (M^{-1} s^{-1})
Native	buffer	7.5	1380 ± 70	0.247 ± 0.048	$5.589 \times 10^{+6}$
	AOT ($W_0 = 5$)	5.0	6.2 ± 0.68	0.801 ± 0.171	$7.511 \times 10^{+3}$
Immobilized	buffer	6.5	1250 ± 110	0.751 ± 0.022	$1.661 \times 10^{+6}$
	AOT ($W_0 = 5$)	5.0	4.63 ± 0.44	0.605 ± 0.113	$7.603 \times 10^{+3}$

Conditions: 30 ± 0.1°C. (A) in aqueous systems: Mes buffer 0.1 M, pH 6.5 or Hepes 0.1 M, pH 7.5; (B) in micelles: [AOT] = 0.1 M; Acetate buffer 2.2 mM, pH 5.

The microgel decreased the $K_{m,app}$ value by 25% and the k_{cat} value by 23%. Factors that may give rise to this kinetic effect in the protein as a consequence of the linkage to the microgel were investigated below.

4.2. Fluorescence study of the enzyme in water and in micelles

A comparative study of the emission fluorescence of the conjugated and native enzymes was carried out in water and in micelles. Most of the isospecies of *Candida rugosa* lipase have 5 Trp [4]. The protein spectrum of isolipase B was dominated by tryptophan's absorbance and emission ($\lambda_{max} = 333$ nm). Table 2 shows the wavelength maxima and emission intensities of lipase tryptophans in aqueous and micellar ($W_0 = 5$) systems. The emission of the enzyme was blue shifted in the buffer medium when immobilized. It displayed a more apolar environment of its Trp. Experiments of circular dichroism spectroscopy using lipase–microgel were not successful, due to the high interference produced by the polymer. But, the blue shift of the mean emission of the 5 Trp of this lipase was enough to prove the protein conformational change, considering that the Trp of this protein are not accessible to the solvent [4]. Similarly to the effect of the microgel on the kinetics results ($k_2^0 = k_{cat}/K_{m,app}$ in Table 1), its effect on the Trp emission was opposite in buffer and in micellar systems: the lipase–microgel emission was red shifted in micelles.

4.3. Structural characterization of the microgel and of the micellar aggregates in the absence and presence of microgel

A physico–chemical characterization of the enzyme–micelle systems was carried out in order to clarify some aspects of the microgel effect on the biocatalysis in AOT micelles.

4.3.1. Determination of the microgel size

The hydrodynamic radius of the crosslinked particles determined by dynamic light scattering at 30°C was found to be 400 ± 20 Å and showed a low polidispersity.

4.3.2. Water activity of the micellar aggregates

The relevance of the micellar hydration degree and the free water content on enzyme activities has been demonstrated [17,19,20,22]. In consequence, the water activities (A_w) of different micellar aggregates ($W_0 = 5$, 12.5 and 30) in the presence of the lipase or the support were studied. Results in Table 3 showed that A_w of the empty nanodroplets rose with increased W_0 (parameter related to the droplet size). These values were in accordance with those reported for the AOT micelles in hexadecane, dodecane, octane, etc [32,33]. The protein requires a certain number of water molecules for its solvation, and it should decrease A_w of the aqueous nanodroplets. This effect was only significant at W_0 values between 12.5–30, where the existence of a pool with free water in

Table 2
Wavelength and intensities maxima of the native and immobilized lipase in water and micelles

Enzyme	[Protein] (μM)	System	λ_{max} (nm)	I_{max} (AU)[a]
Native	0.40	buffer (pH = 7.5)	333.0	70
Immobilized	0.40	buffer (pH = 6.5)	328.5	69
Native	0.28	AOT ($W_0 = 5$, buffer pH = 5.0)	333.0	206
Immobilized	0.28	AOT ($W_0 = 5$, buffer pH = 5.0)	336.0	296

Conditions: $\lambda_{ex} = 295$ nm and $\lambda_{em} = 305$–450 nm; 30 ± 0.1°C; (a) in water: [buffer] = 0.1 M; Emission and excitation slit widths were 2.5 mm (b) in micelles: [AOT] = 0.1 M; water or buffer 1.5 mM; Emission and excitation slit widths were 5 mm.
[a]AU: Arbitrary units.

Table 3
Influence of the protein and microgel on the water activities of micelles and microemulsions

W_0	pH_{opt}		A_w^a
5.0	5.0	—	0.88
5.0	5.0	(+ microgel)	0.92
5.0	5.0	(+ Enz)	0.88
12.5	5.5	—	0.97
12.5	5.5	(+ Enz)	0.95
30.0	5.5	—	1.00
30.0	5.5	(+ microgel)	1.00
30.0	5.5	(+Enz)	0.96

Conditions: 30 ± 0.1°C, [AOT] = 0.1 M; [Buffer] = 2.2 mM; when indicated the system contained the protein (8.32 nM) or 5 μl of microgel
[a] In all the cases the experimental error was lower than 3% of the A_w value.

empty micelles has been reported [34]. Table 3 showed that the native protein has its essential water to work, but it was not totally solvated at the W_0 value of optimal activity ($W_0 = 5$). The amount of water used for its solvation was low and did not reduce A_w at $W_0 = 5$.

Free water molecules are involved in the microgel solvation sphere, but A_w in the droplets containing the microgel was higher than A_w of the empty micellar system, at $W_0 = 5$. Moreover, there was enough free water to solvate the microgel, the enzyme and the tensioactive at the highest W_0 studied ($W_0 = 30$) and thus $A_w = 1$. These A_w data in the presence of microgel can be only explained on the basis of the redistribution of the micellar assemblies giving rise to larger droplets, which include the crosslinked polymer. It must be considered that the water pool radius of the empty micelles (18 Å ($W_0 = 5$) and 55 Å ($W_0 = 30$), obtained by small angle X-ray scattering [35]) were much smaller than the radius of the water soluble acrylic microgel (400 Å). An additional probe of the placement of the microgel into the droplet was obtained in the next fluorescence study with NOH and NDSOH (see Section 4.3.3).

4.3.3. Fluorescence study of probes solubilized in different regions of the micelle

The acid–base behaviour changes as a function of the water content, depends on the residence site of the acid (base) in the micelle, and on the distance from the polar head layer [36]. NOH and NDSOH may undergo protolysis in the excited state and were thus used as adequate probes for the study of the acid-base behaviour of water molecules forming the Stern layer and the water core, respectively.

Table 4 shows the wavelength maxima and intensities of NOH in AOT micelles ($W_0 = 5$) in the presence and absence of microgel. The characteristic band of the basic form NO^{-*} did not appear in these systems [37,38], because the environment of the probe was the interfacial region which was more polar that n-heptane, but not polarizable enough to cause proton transfer from NOH to water [36]. A blue shift and decrease in the extinction coefficient had been found for NOH when the micellar size was increased [36]. These two effects on the spectrum of this probe were obtained when the microgel was added (Table 4), which was in agreement with the greater volume of the micelles containing the crosslinked polymer.

NDSOH was solubilized in the aqueous micellar pool, due to the electrostatic repulsion between the anionic heads of the AOT and the negative groups of the probe [36]. Changes in the fluorescence spectrum of NDSOH, as a consequence of the different properties of the aqueous micellar region were investigated (Table 5). In AOT micelles, the emission of NDSOH had two bands corresponding to the acidic and basic forms of its excited state. The microgel modified the properties of the aqueous envi-

Table 4
Influence of the support on the wavelength and intensities maxima of 2-naphtol in micelles

Microgel content (μl)	λ_{max} (nm)	I_{max} (AU[a])
—	356.0	965
1	355.5	813
6.9	355.0	779

Conditions: $\lambda_{ex} = 315$ nm and $\lambda_{em} = 320$–550 nm; emission and excitation slit widths were 2.5 mm; 30 ± 0.1°C; [AOT] = 0.1 M; $W_0 = 5$; acetate buffer 1.5 mM; [2-naphtol] = 12.5 μM.
[a] AU: Arbitrary units.

ronment of NDSOH, shown by the red shift of the two emission bands of NDSOH in the presence of this polymer. The micellar aqueous pool became more polar in the presence of the microgel. These results can be only explained considering that the polymer is placed into the aqueous micelle.

4.4. Effect of the microgel on the lipase stability in aqueous and micellar systems

The inactivation curves of the native and immobilized lipases incubated in water and micellar media at their respective optimal pH's and 30 ± 0.1°C were obtained. The incubation times at which the enzyme activity was 50% of its initial value ($t_{50\%}$) are shown in Table 6.

The linkage of the lipase to the microgel reduced its stability in water. The different stabilities of the native and immobilized lipase B in water could not be attributed to the distinct pH's of the buffer solutions (pH 7.5 and 6.5, respectively), because $t_{50\%}$ of the native lipase decreased when the pH rose in this pH range [14]. The stability decay was related to the enzyme–microgel interaction, observed in the lipase fluorescence study (Table 3).

In micelles, the stability of the immobilized lipase showed an opposite dependence on W_0 to that of the native lipase. The stability of the immobilized lipase decreased when W_0 diminished.

The enzyme–polymer linkage decreased the stability of the protein in water by 3.7 times. The effect of the micellar medium on the enzyme stability was negative and the enzyme–micelle interactions decreased the stabilities of both, lipase and lipase–microgel by an order of magnitude.

Table 5
Influence of the support on the wavelength and intensities maxima of 1-naphthol-6,8-disulphonate, NDSOH, in micelles

Microgel content (μl)	λ_{max} (nm)	I_{max} (AU[a])
—	375.5	52
	456.5	924
1	377.0	66
	457.0	987
4	377.0	60
	457.0	992
15	379.0	58
	457.0	992

Conditions: $\lambda_{ex} = 310$ nm and $\lambda_{em} = 350$–600 nm; emission and excitation slit widths were 2.5 mm; 30 ± 0.1°C; [AOT] = 0.1 M; $W_0 = 5$; acetate buffer 1.5 mM; [NDSOH] = 47 μM.
[a]AU: Arbitrary units.

Table 6
Stabilities of the native and immobilized *Candida rugosa* B lipases in aqueous and micellar systems

Enzyme	System	pH[a]	$t_{50\%}$ (h)[b]
LB	aqueous	7.5	26 ± 1
	AOT ($W_0 = 5$)	5.0	2.0 ± 0.5
	AOT ($W_0 = 12.5$)	5.0	0.9 ± 0.1
	AOT ($W_0 = 30$)	5.5	0.4 ± 0.1
LB-microgel	aqueous	6.5	7 ± 2
	AOT ($W_0 = 5$)	5.0	0.10 ± 0.05
	AOT ($W_0 = 12.5$)	5.5	0.20 ± 0.02
	AOT ($W_0 = 30$)	5.5	0.40 ± 0.06

Conditions: (A) in aqueous systems: [biocatalyst] = 0.16 g/l; Mes buffer 0.1 M, pH 6.5 or Hepes 0.1 M, pH 7.5; (B) in micelles: [biocatalyst] = 0.041 g/l; [AOT] = 0.35 M; buffer 5.25 mM. Other conditions: 30 ± 0.1°C.
[a]The optimal pH value of the enzyme in the *p*-nitrophenylbutyrate hydrolysis.
[b]Time at which the enzyme has 50% of its initial activity.

5. Discussion

Native lipases are placed into the aqueous micellar aggregate and work at the micellar interface where their active centres contact the apolar substrate (here PNPB). This ester is partitioned between the continuous organic phase and the interface. The anionic micellar interface of AOT systems causes an opposite effect on the pH,opt of surface active lipases than on the pH_{opt} of non-surface active enzymes. Thus, this micellar system shifts the optimal pH's of different lipases to lower values than in water [22,23], while non-surface active enzymes like α-chymotrypsin [39], tyrosinase [40], alkaline phosphatase [41], etc., show shifts to the basic region of pH. These opposite shifts in the opti-

mal p values have been attributed to the different localization of their active centres into the micelle regions. Thus, changes in the dielectric constant of the environment of the active centres should produce different shifts in the pK_a values of their amino acid residues. Here, the micelles decreased the optimal p values of both, the native and the immobilized lipases. The similar variation of their pH,opt with W_0 value and their similar catalytic efficiencies in micelles suggested that the immobilized lipase was also working at the interface in this hydrolytic reaction. These two facts suggested that the active site is properly oriented to interact with the micellar interface when its is immobilized.

C. rugosa produces various closely related isoforms (84% sequence homology considering the whole family, [42]. The peak of the mean emission of the Trp of the native lipase B in micelles was previously found at the same wavelength as in water and was attributed to the low penetration degree of this lipase B into the micellar interface where it works [22]. This spectral behaviour of isoenzyme B, is further evidence for the aqueous environment of most of the protein molecule in the AOT nanodroplet (Fig. 3A). The higher interaction of *C. rugosa* lipase A with the apolar region of the micellar interface produced a blue shift of its emission spectrum [22]. In contrast, the fluorescence spectra of this work showed the higher polarity of the Trp environment of the immobilized protein (isolipase B, Table 2). This would be related with the fact that the microgel increases the polarity of the aqueous micellar pool (Table 5). These changes on the protein and on its aqueous environment gave rise to the kinetic changes of Table 2.

Besides the pH studies also kinetic results, such as the slight increase of the catalytic efficiency ($k_{cat}/K_{m,app}$) in micelles due to its immobilization, supported that the active site of the immobilized lipase was oriented towards the micellar interface (Fig. 3B). Moreover, while the different penetration of *Candida rugosa* isoenzymes A and B in the micellar interface

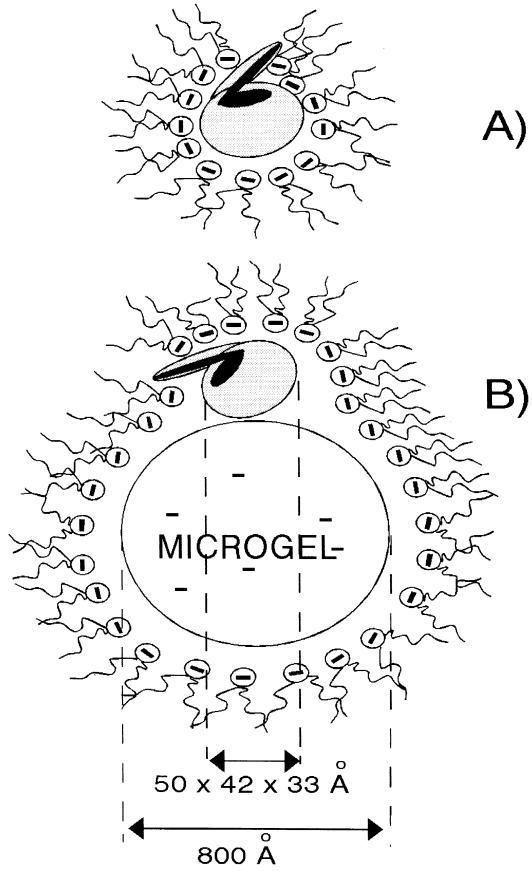

Fig. 3. (A) Lipase in micelles; (B) lipase–microgel in a reorganize micellar aggregate. The interaction between the interface and the active site of the native and immobilized lipases. Lipase dimensions were calculated from the structural data [11] using graphics program O [46].

gave rise to changes of activity of several orders of magnitude [22], the increase of the micellar volume due to the microgel did not change the order of magnitude of the lipase B activity ($k_{cat}/K_{m,app}$). This may be explained considering that the lipase solubilization region (more precisely the penetration degree of the lipase into the micellar interface) was not significantly varied in the presence of this support. This means that neither the penetration of the enzyme into the interface increased, nor the distance from the enzyme to the micellar interface was significantly enhanced and thus, the enzyme–interface interaction was not substantially altered (Fig. 3B).

The enzyme–microgel linkage gave rise to opposite variations in the kinetics and spectrofluorimetric results in water and in micelles (Fig. 3B). The variations in the kinetic parameters in micelles in the presence of microgel were due to the increase of the micellar size, the higher polarity of the micellar water and the protein changes responsible for the different Trp environment. The results made evident the relevance of the enzyme–surfactant and the enzyme–microgel interactions.

There is an on going discussion in the literature about a possible relation between the enzyme size and the optimal size of empty micelles (related to W_0), for the catalysis. This work showed that in the case of lipases the increase of the biocatalyst size by the microgel linkage at constant hydration degree (W_0) did not change the optimal W_0 value ($W_0 = 5$).

The stability of the native lipase (Table 6) was lower in any micellar solution than in buffer, because the interaction of the lipase with the interface is negative for the stability of these lipases. Native isolipase B is solubilized into the water core of AOT systems but maintaining the necessary contact with the micellar surface and it resulted more stable in micelles of small size [22]. The higher stability of enzymes which do not penetrate the interface in smaller than in larger droplets had been associated with the increase of the enzyme rigidity in the small aggregates [43] and it seemed to happen also in this case. The reduction of A_w in the micellar medium also increases the stabilities of proteins [44,45]. Free water molecules act in chemical processes of proteins inactivation, such as hydrolysis of the peptide bonds, etc. In accordance with this, the $t_{50\%}$ values of the native lipase B decreased when W_0 increased [22]. The opposite behaviour was found with the lipase–microgel derivative, and its stability increased at higher W_0 values (Table 6). The interactions between the enzyme and the microgel, which decreased the lipase stability in water, must be higher when W_0 decreased and, thus, the negative effect of the microgel on its stability was more important at small W_0 values. Some of the changes detected in the protein environment also should affect the stability of the immobilized lipase in the micellar medium. The formation of bigger aggregates in the presence of the microgel, increased A_w of micelles of small W_0 (5) and the free water molecules involved in the different chemical processes of enzyme denaturation. Moreover, the simultaneous change of the shape and size of the micellar aqueous pool due to the microgel (Fig. 3B), could minimize the micellar effect on the enzyme rigidity. Fig. 3B is a schematic diagram showing how the conformational mobility of the enzyme in the aqueous phase is not as restricted as it may be in Fig. 3A.

In nature, a similar charged interface may be also found in processes with dissociated fatty acids. The enzyme–microgel immobilization method described here may have possible applications in industrial processes. Due to the increment of the molecular weight of the enzyme–microgel derivatives, these may be used in membrane reactors, using membranes with large pore sizes which allow higher flow rates.

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