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Activity decay and conformational change of lipase in presence of organic solvents. A fluorescence study of *Candida rugosa* lipase

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Abstract

Different factors that modify the stability of *Candida rugosa* lipase in presence of organic solvents have been investigated. Distinct aqueous and, mono- or biphasic organic media have been studied comparatively. Stabilities of crude and purified isospecies from *Candida rugosa* lipase in a polar organic medium have been compared, and the effect of contaminants and depressors of water activity discussed. In addition, factors typically tested in studies of enzyme activity have been considered: water content, solvent properties, protein aggregation, enzyme concentration, etc.

The crude lipase suspended in acetonitrile is more stable than in n-heptane with the same water content, but the opposite is true when the protein is solubilized in these organic media–water systems. Similar changes of enzyme conformation during the inactivation process of crude and pure lipase, in acetonitrile–water media and in water have been detected by fluorescence spectroscopy. Inactivation curves in presence of acetonitrile are biphasic. The first activity decay was found to be favored by acetonitrile, but the fluorescence study reflects that conformational changes causing the rest of the inactivation are decelerated in presence of this co-solvent.

Keywords: *Candida rugosa*; *Candida cylindracea*; Fluorescence ; Lipase; Organic media; Stability

1. Introduction

Before the last decade microbial lipases were mainly used in pharmaceutical industry for commercialization of digestive preparatives. There are different patents to use lipases in the preparation of fatty acids and glycerol. Specific lipases are used in oil modification and to prepare aromas and detergents [1]. Lipase from *Candida cylindracea*, recently named *C. rugosa*, is used in hydrolysis of oils for the production of soaps [1].

In the future, an important use of lipase in the industry is expected. Lipases have been more studied than other enzymes in organic media for synthetic purposes [2–7], due to their perspectives. Their potentiality in the resolution of racemic mixtures of optically active acids and alcohols [2], regioselective hydrolysis of esterified carbohydrates [3], and esterification of fatty acids with glycerol [3,4], alcohols and sugars [5–7] has been increased in recent years.

Lipases (EC 3.1.1.3) are a group of enzymes which in vivo catalyze the hydrolysis and synthesis of triglycerides, producing or consuming fatty acids [8]. Their activity is greatly increased at the lipid–water interface [9]. Their interfacial activation has been early associated with a conformational change in the protein molecule [10,11].

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The structure of different lipases have been recently elucidated: *Rhizomucor miehei* [12], human pancreatic lipase [13], *Geotricum candidum* [14] and *Candida rugosa* [15]. In these lipases, the catalytic triad is not exposed to the solvent, but it is instead covered by short amphiphatic α-helix [16–18]. In fact, it is the conformational change in these enzymes, when they come into contact with the water–lipid interphase, that is regarded as an essential part of their lipolytic function.

Purification of Lipase crude from *Candida cylindracea*, recently named *C. rugosa* lipase, has been studied previously [19–22]. We obtained and characterized two purified isoenzymes (lipase A and B). These two lipases have the same molecular mass (60 kDa), similar amino acid content and N-terminal sequences, but they differ in hydrophobicity, neutral sugar content and number of isoforms. Lipase A is a single protein (p*I* = 5.5) but lipase B is a mixture of four isoforms which have different p*I*s ranging from 4.8 to 5.0, and probably different neutral sugar content [21]. In addition, lipase A showed a higher esterase character in aqueous and micellar media, while lipase B is a better catalyst for lipase substrates [19–21]. These two lipases differ in their stability in water to pH and temperature. cDNA clones for these lipases have been recently isolated and their nucleotide sequences determined: lipases A and B were found to be 79% identical at the amino acid level [23].

CCL [1] lipase has become one of the more studied lipases in organic media. The stability of enzymes in organic solvents, in optimal conditions, is higher than in water. In the case of lipases, Zaks and Klibanov, showed their high stability (more than 12 h at 100°C) in organic media [24]. The water content and solvent nature have an important effect in the stability of enzymes in organic media. In general, it is considered that enzymes in hydrophobic solvents present their highest stability [25]. The main factor is not so much the solvent–protein interactions per se, but those with water on the enzyme. An exception are formamide and dimethylsulfoxide, where significant specific interactions between groups of the solvent and protein occurs [26].

The potentiality of lipases and other enzymes in organic media has been extensively proved. Regio- and stereoselective processes catalyzed by lipases, will enable the obtention of different esters with pharmacological properties. However, there are still fundamental questions which must be answered. Much has been done to study the influence of factors (i.e. solvent nature, its water content, aggregation degree, enzyme concentration, etc.) in the kinetics of enzyme catalyzed reactions. However, their industrial application requires high enzyme activity, but also good stability. Thus, the influence of different organic media in enzyme stability is an important factor to investigate. Most of the stability studies reported in the literature are under the conditions of each particular process or study. Thus it is difficult to extract comparative conclusions from the reported data.

In this work we have done a systematic study of different factors, that modify the stability of *C. rugosa* lipase in presence of organic solvents. Aqueous and organic media, mono- and biphasic, have been studied comparatively. In addition, the stabilities in a polar organic medium of crude and purified isospecies from CCL have been compared, and the effect of contaminants and depressors of water activity discussed. Factors typically checked in enzyme activity studies have been considered looking at their role in the enzyme stability. General conformational changes of lipase solubilized in water–polar co-solvent media have been investigated by fluorescence spectroscopy, during its inactivation process.

[1] CCL, crude lipase from *Candida cylindracea or C. rugosa*; CCL A and B, isoenzymes A and B obtained after purification of CCL; pNPB, *p*-nitrophenyl butyrate; TB, tributyrin; PEG, methoxypolyethylene glycol, MW 5000; NaPi, Na_2HPO_4/NaH_2PO_4 buffer solution; ACN, acetonitrile; $t_{50\%}$, time to lose 50% of the enzyme activity under given conditions; *f*, stabilization factor, in conditions 'a' with respect to conditions 'b' is the relation: $f = t_{1/2}(a)/t_{1/2}(b)$; Lp of a solvent, logarithm of its partition coefficient between octanol–water two liquid phase system; D/L_{MB}, the ratio flask diameter/length of magnetic bar; A_w, water activity.

2. Materials

Candida cylindracea lipase Type VII, *p*-nitrophenyl butyrate, *p*-nitrophenyl acetate, tributyrin, and methoxypolyethylene glycol (Mr 5000), were used from Sigma; D(−)-fructose and D(+)-lactose from Merck, D(+)-galactose from Aldrich, and D(−)-sorbitol from Sigma. n-Heptane and acetonitrile, HPLC quality, were purchased from Scharlau. Isopropylic ether was from Carlo Erba. All the solvents were previously dried with molecular sieves with an effective diameter of 4 Å from Sigma, contained less than 0.01% (w/w) of water (determined by the Karl Fisher method). Phenyl Sepharose CL-4B and Sephadex G-25 were from Pharmacia. Tris buffer was from Scharlau and mono- and di-sodium phosphates from Panreac.

3. Methods

3.1. Purification of Candida cylindracea lipase

Purification of two extracellular lipases from CCL was carried out according to the method previously reported [20]. 7 g of crude powder were suspended in 70 ml of 0.25 M NaPi buffer, pH = 7.0. After 90 min stirring at 0°C, it was centrifuged at 17000 × g for 20 min at 4°C. The supernatant was loaded on the first column.

The obtained fractions containing isoenzymes A and B [19,20] were concentrated separately using an ultrafiltration membrane Amicon PM 30. This process was done in an ice bath to avoid the loss of the proteins activity. Concentrated fractions were freezed at −70°C and lyophilized overnight in a lyophilizer by Labconco. Lyophilized isoenzymes were stored at −20°C until their use. The protein content and specific activities of crude and purified lipases were,

CCL (11% w/w protein)

$= 0.707 \pm 0.041$ (10^{-3} Kat/mg protein)

CCL A (63% w/w protein)

$= 3.58 \pm 0.09$ (10^{-3} Kat/mg protein)

CCL B (97% w/w protein)

$= 5.26 \pm 0.13$ (10^{-3} Kat/mg protein)

3.2. Incubation of CCL in aqueous media

In the study of influence of ACN in the stability of CCL in water, several flask containing a variable percentage of ACN (3, 5, and 15% v/v) were prepared. Every flask contained 200 mg of crude CCL in 50 ml of buffer solution 1 mM NaPi, pH = 7.2. All the incubations were thermostated at 30°C, and stirred at 100 rpm.

3.3. Incubation of CCL in acetonitrile

Several flask of 100 ml, containing 200 mg of lipase powder in 50 ml ACN, and the corresponding percentage of buffer solution 1 mM NaPi, pH 7.2 were prepared. These enzymatic suspensions were incubated at 30°C, and stirred at 100 rpm The relation flask diameter/length of magnetic bar (D/L_{MB}) was 1.8. Residual activity of CCL was determined at distinct incubation times.

After the indicated incubation time, a portion of the enzyme powder was extracted and vacuum dried. 4 mg of dry enzyme was used in the standard reaction to determine its enzyme activity.

In the study of the influence of Lp of the medium in the CCL stability, 200 mg of lipase were incubated in 50 ml of solvent, containing 1% (v/v) of 1 mM NaPi pH 7.2. The value of D/L_{MB} was 2.25.

3.4. Incubation of CCL in n-heptane

CCL stability in n-heptane was studied incubating several flask of enzyme suspension for each water content studied. Every flask (20 ml) con-

tained 4 mg of lipase in 2 ml of n-heptane and the corresponding percentage of 1 mM NaPi buffer, pH 7.2. Incubations were carried out at 30°C, and 100 rpm (ratio $D/L_{MB}=3$). The residual activity of CCL was determined at distinct incubation times.

In the study of the influence of Lp of the medium in the CCL stability, different flask ($D/L_{MB}=3$) containing 16 mg of lipase were incubated in 4 mL of n-heptane with 1% (v/v) of 1 mM NaPi pH 7.2.

3.5. Incubation of CCL in solvents mixtures

The influence of Lp of the organic medium in the activity and stability of CCL was done using solvents mixtures. Mixtures ether–heptane were studied in proportions 30:70, 70:30 (v:v). These enzyme incubations contained 16 mg in 4 ml of solvent, and the ratio D/L_{MB} was 3.

Lower values of Lp were obtained with ether–acetonitrile mixtures, in proportions 30:70, 70:30 (v:v). In this case 200 mg of lipase were incubated in 50 ml of solvents mixture, with a ratio $D/L_{MB}=2.25$.

Lp of solvents mixtures were obtained from equation:

$$\mathrm{Lp(mixture)}=x_A\mathrm{Lp(A)}+x_B\mathrm{Lp(B)}$$

where x_i and $\mathrm{Lp}(i)$, are the molar fraction and Lp of the solvent i, respectively.

The different mixtures contained 1% (v/v) of 1 mM NaPi buffer, pH 7.2.

In the case of non-water soluble media ($\mathrm{Lp}\geq 1.9$) several flask were prepared, using the same procedure employed for pure n-heptane. In media of lower Lp value, all the lipase amount required was incubated in a flask, using the same procedure employed in acetonitrile.

3.6. CCL incubation in presence of additives

The presence of methoxypolyethylene glycol in the stability of CCL in n-heptane was studied. Different flask of 25 ml, containing 8 mg of enzyme powder in 4 ml of n-heptane, 8 mg of PEG, and 1% (v/v) of 1 mM NaPi buffer, pH 7.2 were prepared. Incubations were kept at 30°C and stirred at 100 rpm (ratio $D/L_{MB}=3$).

Residual activities were determined at distinct incubation times.

3.7. Stability of isolipases CCL A and B in ACN

The stability of CCL A and B, was determined incubating 2.6 and 4.1 mg of isolipase A, and 2.6 mg of CCL B, respectively, in 1 ml of acetonitrile. The incubation media contained 1 and 1.5% (v/v) buffer solution in the case of CCL A and B, corresponding to their optimal water content for monoolein synthesis, respectively [27]. All the incubations were kept at 30°C and 100 rpm.

3.8. Determination of residual activities of incubated lipases

In case of non water soluble incubating media, a volume (ml) of 1 mM NaPi buffer, pH 7.2, equal to the mg of lipase was added to the incubation medium. The mixture was kept stirring for 4 min. After separation of phases, 50 μl of the aqueous phase were extracted, and used in the standard reaction.

In the case of water soluble incubation media, 4 mg of dried enzyme were added to 4 ml of 1 mM NaPi buffer, pH 7.2. After stirring for 4 min, 50 μl were used to determine the residual activity of CCL.

The time for half inactivation of the isoenzymes ($t_{50\%}$) in a given conditions, has been defined as the necessary incubation time to lose 50% of its initial activity in the standard reaction, pNPB hydrolysis.

The stabilization factor (f) in conditions 'a' respect conditions 'b' is the ratio

$$f=t_{50\%}(\mathrm{a})/t_{50\%}(\mathrm{b})$$

3.9. Standard reaction for determination of CCL hydrolytic activity

The same standard reaction for CCL activity determination was used in all cases. The standard

reaction mixture was 0.57 mM of pNPB substrate solution in 0.1 M NaPi buffer, pH 7.2. Reactions contained 3% (v/v) acetonitrile. For each assay the non-catalyzed reaction rate was measured at 30°C and 400 nm, for 5 min. An UV–vis spectrophotometer UVIKON 930 of Kontron Instruments doted with magnetic stirrer and thermostated cell was used. The enzyme catalyzed hydrolysis was started by addition of 50 μl of enzyme solution. The rate of the lipase-catalyzed reaction was obtained by substraction between the total reaction rate and the non catalyzed hydrolysis rate (less than 5% of total reaction).

The specific activities of isoenzymes A and B from CCL were determined in a pH-stat Radiometer, measuring the hydrolysis of tributyrin. These reactions were carried out in 1 mM Tris buffer, pH = 7.0, containing 3% (v/v) acetonitrile, at 30°C. The specific activity was obtained considering the protein content determined by the Peterson's method [28].

3.10. Fluorescence study of CCL in aqueous-acetonitrile media

Fluorescence spectra of purified lipase B and crude CCL in 0.1 M NaPi buffer, pH = 7.0, in presence or not of 3% (v/v) ACN were recorded at different incubation times. Protein concentration in the cuvette was 0.0125 mg/ml and 0.1 mg/ml (100% w/w of protein), respectively. Spectra were made at 26°C at 280 nm excitation and 450–280 nm emission, in a Perkin Elmer fluorescence spectrometer LS 50B. Spectra were uncorrected for the instrument sensitivity, but Raman emission of the solvent was subtracted.

4. Results

Industrial application of enzymes in processes in organic media needs to be developed. The stability of biocatalysts is a key factor in biotechnological processes. Thus, the stabilization of enzymes is a primary objective to allow their industrial use. Factors like solvents, additives, etc. must be controlled.

4.1. Stability of CCL in aqueous media

In aqueous media, the low solubility of hydrophobic substrates may be increased using water miscible co-solvents, that reduce the dielectric constant of the system. In the case of lipases, polar co-solvents are used in reactions of fats with low melting point. They are also used in hydrolysis of liquid triglycerides and non-natural liquid substrates, to obtain a homogeneous reaction mixture – emulsion, dispersion, etc. – to enhance reproducibility. In some cases, tensioactives are employed. However, in general, polar co-solvents reduce the life of biocatalysts [29].

In a previous study of CCL [30] its maximum stability was found at neutral pHs (6.5–7.5), that decreases when temperature increases. The inactivation of CCL at the water–air interphase has been described [31].

In the present work the effect of distinct proportions of a polar co-solvent, ACN has been investigated. The stabilities of crude CCL in 1 mM NaPi buffer, pH = 7.2 at 30°C, in absence and presence of a variable percentage of acetonitrile (3, 5 and 15% v/v) are represented in Fig. 1. In presence of this co-solvent, the activity decay of crude CCL is faster than in its absence. The negative effect is more important at higher percentages of added ACN. Direct comparison of $t_{50\%}$ in absence and presence of 15% of ACN, shows that CCL is 22 times more stable in aqueous medium without co-solvent.

4.2. Stability of CCL in acetonitrile

Stability studies of enzymes in nearly anhydrous media have emerged as a consequence of their use in organic synthesis. In biocatalytic processes it is necessary to consider the enzyme and reaction requirements. Use of solvents of a given polarity may be necessary for some substrates. But, the success of the process will be conditioned to achieve high enzyme stability in the medium.

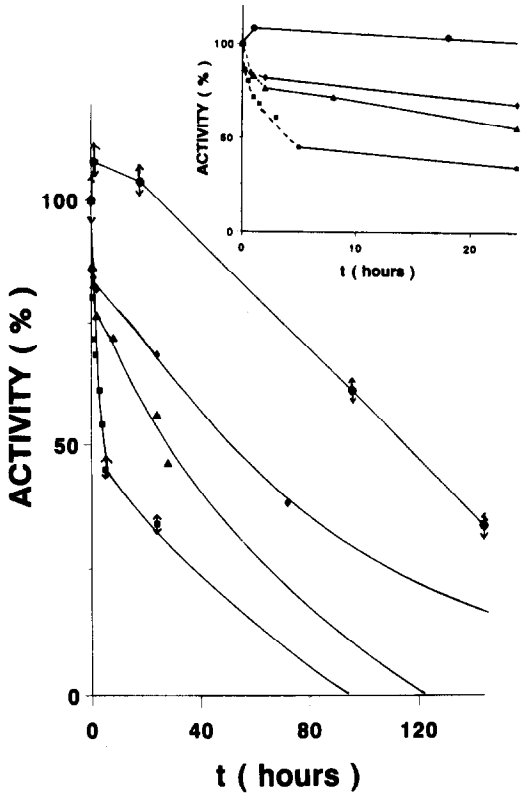

Fig. 1. Stability of crude *Candida rugosa* lipase in aqueous media with distinct contents of acetonitrile. Conditions: [CCL] = 4 mg/ml, [NaPi] = 1 mM, pH 7.2 and 30°C. (●, 0% (v/v) ACN; ♦, 3% ACN; ▲, 5% ACN, and ■, 15% ACN). 100% of activity without ACN corresponds to 0.386 ± 0.015 mol/s. Insert shows the biphasic shape of curves, each one represented by dashed and solid lines, respectively.

The study of CCL stability in acetonitrile systems with different water content (1 mM NaPi buffer, pH = 7.2) is presented in Table 1.

This table shows a value of the optimal aqueous content of 3% v/v, where lipase has a half inactivation time of 212 h. When the percentage of added water increased, the stability of crude enzyme diminished, being much lower than in water ($t_{50\%}$ (in water) = 114 h) at buffer amounts higher than 5%. However, the lipase stability in this solvent hydrated with the optimal percentage was nearly twice higher than in the buffer solution.

4.3. Stability of CCL in n-heptane

The use of apolar solvents like reaction media in organic synthesis is necessary in the case of biotransformations of hydrophobic compounds.

The study of the influence of the aqueous content (1 mM NaPi buffer solution, pH = 7.2) in the stability of crude CCL in n-heptane, is represented in Fig. 2.

In Fig. 2A the maximum stability was found at 0.5% v/v of buffer solution. In this conditions the lipase resulted 4.4 times more stable in n-heptane ($t_{50\%} = 500$ h) than in an aqueous solution of the same pH ($t_{50\%} = 114$ h). At values higher than the optimal aqueous content, the stability decreased continuously. The lipase stability tends to be similar to stability in buffer solution, at buffer contents higher than 3% v/v, (Fig. 2B).

The agitation type used for incubation in n-heptane was less effective ($D/L_{MB} = 3$) than used in ACN ($D/L_{MB} = 1.8$). Thus, the two studies cannot be directly compared. With that aim, a comparative study between distinct solvents has been also carried out.

4.4. Stability of CCL in solvent mixtures

Physical properties of the solvent, such as dielectric constant, water solubility, Hildebrand solubility, etc. have been used to correlate the activity of biocatalysts in different media [32]. However, it has been shown that Lp (logarithm of the partition coefficient of the solvent in the octanol–water two-phase system) gives the best correlations with enzyme activity in organic media [33]. Hydrophobicity and polarity of the solvent are of great importance for enzyme stability. In general,

Table 1
The effect of distinct percentages of 1 mM NaPi, pH 7.2 on the stability of *Candida rugosa* lipase in acetonitrile

NaPi (% v/v)	$t_{50\%}$ (h)
0	97.9 ± 5.2
1	62.5 ± 3.3
3	212 ± 11
5	52.0 ± 2.7
15	4.16 ± 0.22
30	1.00 ± 0.05

Conditions: [CCL] = 4 mg/ml and 30°C. 100% of activity with 3% (v/v) buffer corresponds to 0.318 ± 0.017 mol/s, which is similar to the activity of CCL directly solubilized in NaPi buffer.

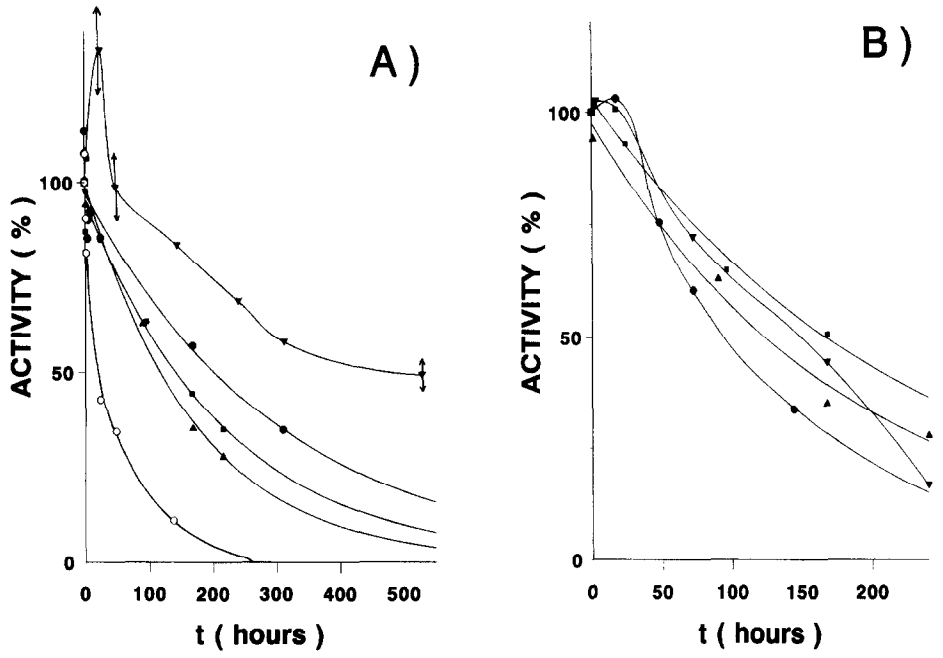

Fig. 2. Stability of crude *Candida rugosa* lipase in n-heptane. Effect of the NaPi content. Conditions: [CCL] = 2 mg/ml, NaPi 1 mM, pH 7.2 and 30°C. 100% of activity in n-heptane (0.5% (v/v) of buffer) corresponds to 0.551 ± 0.047 mol/sec. **A)** (○, 0% NaPi; ▼, 0.5% NaPi; ●, 1% NaPi; ■, 3% NaPi; ▲, 5% NaPi); **B)** (▲, 5% NaPi; ■, 10% NaPi; ▼, 20% NaPi; ●, 100% NaPi).

the more hydrophobic solvents are less harmful to enzymes than the less hydrophobic solvents.

Previous experiments with ACN and n-heptane showed an important effect of the medium polarity in the stability of CCL lipase. A more extended study with organic media of different polarity was done to analyze the effect of their polar nature.

The influence of Lp of the medium in the stability of crude lipase was studied incubating the enzyme in different solvent mixtures, to minimize the effect of other molecular properties of solvents. In all mixtures, the aqueous content was kept constant at 1% v/v. This percentage corresponds to the optimal ratio: water volume/mg of lipase, obtained in Fig. 2A for n-heptane. All the solvents were dried over molecular sieve prior to their use. In these conditions CCL powder was suspended in the solvents mixtures. Results are shown in Fig. 3.

The influence of Lp of the medium in the half inactivation time is represented in a single plot. But the variation of medium polarity was obtained with two distinct mixtures. Consequently, the molecular nature of solvents was different in two intervals. This is the reason why in Fig. 3 the two

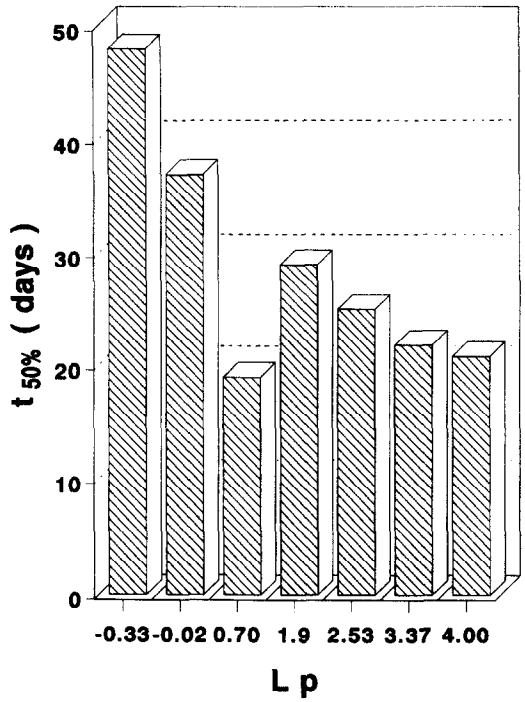

Fig. 3. Stability of crude *Candida rugosa* lipase in solvent mixtures. Influence of Lp of the medium in the time for half inactivation. Conditions: [CCL] = 4 mg/ml, 1% (v/v) 1 mM NaPi, pH = 7.2 and 30°C. The standard error in the determination of $t_{50\%}$ was 0.4–0.8%.

intervals were found to be independent. Also, the resultant organic medium was monophasic (water miscible) for Lp = −0.33 to 1.06 and biphasic for Lp = 1.9–4, and D/L_{MB} value was 2.25 and 3 for mono- and biphasic systems, respectively. However, in each interval a continuous decay of $t_{50\%}$ was observed when Lp increased.

This figure reflected that for the same water content, the stability of dispersed powder of CCL diminished in the all interval (Lp = 0.33–4) when log$P$ of organic medium increased. Acetonitrile with Lp = −0.33, resulted to be the best organic medium. The use of the same D/L_{MB} value would increase these differences. In Fig. 4, crude lipase presented a $t_{50\%}$ value 2.3 times higher in acetonitrile than in n-heptane (Lp = 4) in presence of 1% (v/v) of water content.

4.5. Stability of CCL in presence of additives

Lipase CCL is only active in apolar media [34,35], but Fig. 3 shows that its stability is not so high as in ACN. A possible strategy to improve its stability in apolar media, may be to enhance the structuration degree of water with polar additives. It is known that there is a protector effect by some additives, which increases the enzyme stability in low water content media [36–38]. Gelatin, Carbowax 4000, gum arabic and detergents such as Tween 80 have been shown to protect against thermoinactivation [39,40]. Also divalent cations, neutral salts and polyols [37,41] increase the enzyme stability at high temperatures and in presence of solvents.

Experiments to improve CCL stability in nearly anhydrous media, were carried out as a function of the relevant role of water in the irreversible inactivation found in the previous experiments. With this aim an additive that interacts with water, increasing water structuration and decreasing free water, PEG, has been selected. Its effect in CCL stability was studied in the more negative solvent of Fig. 3, n-heptane. The results are shown in Fig. 4. In this experiment the water content was 1% (v/v), and the ratio protein:PEG was 1:1 w/w. The half inactivation time of incubated lipase

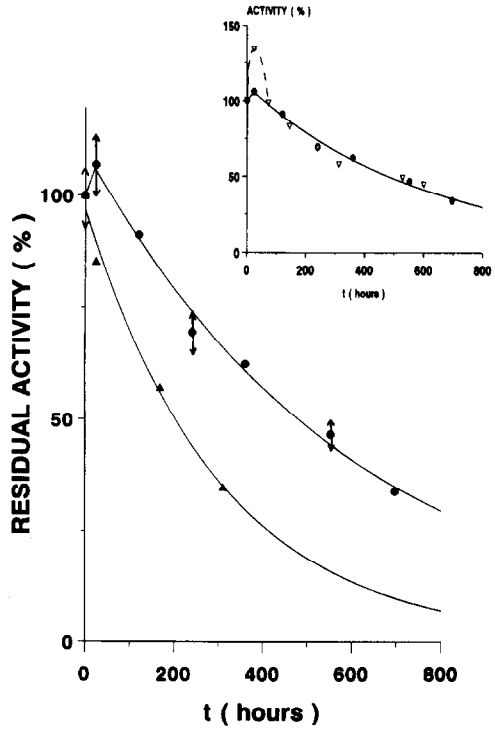

Fig. 4. Stability of crude *Candida rugosa* lipase in n-heptane in presence and absence of additive, PEG. Effect of the addition of 1:1 (w/w) PEG to n-heptane containing 1% (v/v) NaPi 1 mM, pH 7.2 (▲, without PEG; ●, with PEG). Insert: Comparative effect of the hydration degree and additives in stability in n-heptane. Conditions: [CCL] = 2 mg/ml and 30°C. (▽, 0.5% (v/v) NaPi; ●, 1% (v/v) NaPi and 1% (w/w) of PEG).

increased from 180 h. in absence of additive, to 500 h. in presence of PEG. Thus, this additive increased 2.8 times the stability of CCL in n-heptane and 1% (v/v) NaPi buffer.

Inserted in Fig. 4, the stability of crude lipase with the additive and 1% v/v buffer has been compared with the stability without additive and 0.5% v/v of water content. In the two cases the $t_{50\%}$ are found to be similar. The positive effect of this additive is due to its strong interaction with water, reducing the protein solvation, and its chemical action in denaturation processes.

4.6. Influence of agitation type in the stability of CCL

When the enzyme is incubated and stirred in a cylindric reactor, it may suffer additional inactivation due to the protein trituration, when the

Table 2
The effect of the distinct size of magnetic bar on the stability of crude *Candida rugosa* lipase in acetonitrile

Medium	L_{MB} (cm)	D/L_{MB}	$t_{50\%}$ (days)
ACN + 1% (v/v) NaPi	2.0	2.25	41.6 ± 2.2
	2.5	1.80	2.63 ± 0.14
	3.0	1.50	1.58 ± 0.08
ACN + 3% (v/v) NaPi	2.0	2.25	58.5 ± 3.10
	2.5	1.80	9.00 ± 0.47

Conditions: [CCL] = 4 mg/ml, 30°C and 100 rpm.

ratio: diameter of the reactor base/length of magnetic bar (D/L_{MB}), is nearly 1. In the present study it was this type of reactor that was used, but experiments were designed in such a way that the trituration effect were not significant.

The agitation type of the system enzyme–organic medium was studied in acetonitrile. This solvent was selected due to its high interaction with water. Table 2 showed the stability of CCL in ACN and 1% or 3% v/v of water, with magnetic bars of three consecutive sizes (2, 2.5 and 3 cm).

In the two cases, 1% or 3% v/v of water, the stability increased when the size of magnetic bar was reduced. The stability ($t_{50\%}$) of lipase in presence of 1% v/v NaPi increased from 63 h with a bar of 2.5 cm, to 998 h when L_{MB} was 2 cm. This is a factor of 16 times more stable. The stabilization factor increased to 26 times when magnetic bars of 2 and 3 cm were compared. Also, the effect was higher when the hydration of the medium decreased. Thus, in presence of 3% NaPi buffer in ACN, a $t_{50\%}$ value of 226 h with a bar of 2.5 cm was obtained, and 1404 h with a bar of 2 cm (stabilization factor, $f = 6.5$).

4.7. Influence of the enzyme concentration in the stability of CCL

The effect of the enzyme concentration in the process rate – activity – is a factor frequently studied. But, in general its influence on the stability of biocatalyst is not considered. Due to the relationship between protein hydration and its stability in organic media, the influence of enzyme concentration in a medium with constant water content was investigated. n-Heptane was chosen due to its low interaction with water.

The lipase stability increased when the protein concentration did, in presence of 1% v/v NaPi buffer. Thus, its half inactivation time was 216 h and 493 h for enzyme concentrations of 2 and 4 mg/ml, respectively. That is, CCL stability increased when the ratio protein/water did. An aggregation/protection effect of the enzyme at higher protein amounts is also responsible for the enhanced stability.

4.8. Stability of CCL A and B in acetonitrile

The stabilities of purified isoenzymes A and B were studied in ACN. Incubations of each lipase were prepared in optimal reaction conditions for monoolein synthesis at 30°C. Table 3 shows $t_{50\%}$ values for lipases CCL A and B. The stability of purified isospecies in ACN was found to be much lower than that of the crude CCL (Table 1). Similarly to the case of crude CCL, the half inactivation times ($t_{50\%}$) of *Candida rugosa* isoenzymes depend on the amount of the incubated lipase at constant water content. This effect is due to the enzyme aggregation state, and a decrease of the

Table 3
The effect of different polyols in 1:1 weight:weight ratio of enzyme:additive on the stability of isoespecies A and B from *Candida rugosa* in ACN1

Lipase	Additive	$t_{50\%}$ (h)	f[a]
CCL B, 2.6 mg/ml	–	1.4 ± 0.1	1
CCL B, 2.6 mg/ml	PEG (MW = 5000)	6.7 ± 0.5	4.8
CCL B, 2.6 mg/ml	fructose	8.5 ± 0.2	6.1
CCL B, 2.6 mg/ml	galactose	9.5 ± 0.1	6.8
CCL B, 2.6 mg/ml	lactose	8.5 ± 0.3	6.1
CCL B, 2.6 mg/ml	sorbitol	43.2 ± 0.6	31
CCL A, 2.6 mg/ml	–	4.1 ± 0.1	1
CCL A, 4.1 mg/ml	–	23.7 ± 2.5	5.8
CCL A, 2.6 mg/ml	sorbitol	46.7 ± 1.7	11.4

Conditions: 30°C, 100 rpm, 1% and 1.5% v/v of NaPi 1 mM pH = 7.2, for CCL A and B respectively. 10^{-2} Kat corresponds to 4.1 mg/ml of CCL A and 2.6 mg/ml of CCL B.

[a] f represents the stabilization factor, the ratio between the half inactivation times in the two conditions compared.

water molecules free to act in the chemical processes of protein denaturation, when the ratio protein/water increases. When the same weight of lipases were incubated, the stability of isoenzyme A and B was found to be similar (2.9 times higher for the isoenzyme incubated in ACN with 1% (v/v) NaPi than lipase incubated with 1.5% (v/v) buffer). But, when the same activity units – in pNPB hydrolysis – were compared (4.1 mg/ml and 2.6 mg/ml of CCL A and B, respectively), lipase A was found to be 17 times more stable than CCL B.

Due to the low stability of CCL A and B in this medium, the possible effect of polyols like PEG, fructose, galactose, lactose and sorbitol was studied (Table 3). In general, polyols depress the water activity. PEG (MW = 5000) enhanced the stability of crude CCL and purified isoenzymes in nearly anhydrous heptane (Fig. 3) and ACN (Table 3), respectively. This additive increases the structuration of water, decreasing the water activity. Cyclic polyols mono- and disaccharides produced higher stabilization effect in lipase B than PEG 5000. But the highest f value was found with a linear polyol with relatively short chain, sorbitol. It increased by 11.4 and 31 times the stability of lipase A and B, respectively. The stabilized lipases A and B with sorbitol presented similar half inactivation times.

4.9. Spectroscopic studies of Candida rugosa lipase

Fluorescence spectra of *C. rugosa* lipase were obtained to detect possible conformational changes in the protein, during its inactivation in high water content systems. Emission spectra were recorded at different incubation times, during the lipase inactivation. In Table 4 are collected the maxima wavelength and intensities of fluorescence spectra of crude CCL and lipase B. They are dominated by tryptophan's absorbance and emission, in part because Trp has the highest extinction coefficient, and in part because resonance energy transfer from phenylalanine and tyrosine to tryptophan frequently occurs [42]. Thus, lipase spectra showed only emission of Trp at 341–356 nm, but emission bands of Tyr and Phe were not observed. According to reported results, *Candida cylindracea* lipase has 20 Tyr [43] and 5 or 6 Trp [12,23].

The spectra of crude and pure lipases showed important conformational changes during the inactivation process. These conformational changes occur in presence and absence of ACN: initially, the intensity of Trp's peak decreased slightly. After that, a more important enhancement of Trp's intensity was found. The low stability of pure lipase B in water ($t_{50\%} = 30$ hours [20]) shows these spectral changes more rapidly than in

Table 4
Variation of emission spectra of crude and purified *Candida rugosa* lipase with the incubation time. A comparative study of lipase in water–polar and pure water systems

Time (h)	λ_{max} (nm) 0% ACN	I^{a}_{max}	λ_{max} (nm) 0% ACN	I^{b}_{max}	λ_{max} (nm) 3% ACN	I^{b}_{max}
0	332	513	343	228	344	235
0.17	330	491	345	220	344	227
0.33	328	472	345	217	347	223
0.50	328	472	345	213	344	224
1	330	461	346	210	344	227
4	333	443	347	209	343	224
20	332	609	341	388	345	237
44	332	611	338	520	342	319
68	–	–	338	545	337	555

Conditions: [CCL] = 0.1 mg/ml; [CCL B] = 0.0125 mg/ml; NaPi buffer 0.1 M, pH = 7.0; 25°C.
[a] Spectra of pure isolipase B. In presence of ACN, 3% (v/v), the increase of Trp intensity takes place at times longer than 68 h.
[b] Spectra of crude CCL.

the case of crude CCL. Table 4 shows that the intensity enhancement of crude lipase in absence of ACN takes place more rapidly than in its presence (the same effect was found for pure lipase B, not shown). The increase of intensity occurs when the Trp buried in the structure, are oriented to the exterior after protein unfolding.

5. Discussion

The negative effect of ACN in CCL stability in aqueous media (Fig. 1) could be due to its interaction with certain protein groups and/or with water in solution. Considering the high CCL stability in nearly dry ACN (Table 1), the stability decrease in high water content media (3–15% (v/v) ACN in Fig. 1), due to a direct chemical action of ACN with protein may be discarded. Fluorescence spectra in presence of 3–5% (v/v) of ACN, and in pure water (Table 4) showed that the enhancement of Trp's emission of CCL takes place later in presence of ACN. Inactivation curves in Fig. 1 are biphasic (see the insert). Thus, the activity decay in ACN–water systems – initially faster than in water media, and proportional to the ACN content – is a more complex process than conformational changes detected by fluorescence. Our data in Table 4 and Fig. 1 show that CCL inactivation in water–ACN systems occurs after: (i) at first rapid activity decay during a few hours (insert, Fig. 1), and (ii) chemical action of water and/or protein unfolding for more than 80 h:

(i) Fast conformational changes of CCL in water–polar solvent systems, may probably be due to a change in the equilibrium between hydrophobic and hydrophilic intermolecular interactions of protein. It could be produced by the lower solvation ability of water in presence of ACN. In this medium distinct non solvated polar groups in the enzyme may undergo intermolecular interactions, and thus, incorrect structures, but not very different from the native structure, could be easily adopted. To diminish the irreversible inactivation of CCL by conformational changes, Otero et al. [30] developed an immobilization method for this lipase. A multiple covalent linkage of protein to a support gives rise to an insoluble lipase, whose thermostability is 140 times higher than for the native. This stabilization factor (f) was found to be higher in water–polar organic cosolvent systems [30], and it supports the hypothesis that small conformation changes rather than direct ACN–protein interaction produce the initial activity decay.

(ii) When protein is solubilized in an aqueous medium, polar co-solvents reduce A_w and thus chemical action of water in denaturalization processes. In Table 4 the enhancement of Trp intensity was decelerated in presence of ACN. Thus, CCL native conformation is more resistant to hydrolysis of its peptide bonds, partial unfolding, etc. in an aqueous medium with a lower A_w than pure water.

Linear alcohols also produce an important activity decay, and their effect is higher when the alcohol content increases [30]. Fig. 1 shows that the activity decay also occurs with polar co-solvents whose functional group is not the hydroxyl function. Some compounds and/or solvents that are able to interact with water (ACN, ethyl and butyl alcohols, butyric acid, dioxane, etc.), diminishes the stability of CCL in water. Lipase stability in monophasic aqueous media changes with polarity of co-solvent added [30]. This work shows that it is due to its effect on A_w and solvation ability of water. Solvation depends on the water present, but essentially depends of physico-chemical properties of water. Thus, due to the ACN polarity ($Lp = -0.33$), its negative effect is more similar to ethyl alcohol ($Lp = -0.24$) than to the butyl alcohol effect [30].

p-Nitrophenyl esters and apolar substrates that may be solubilized in water with cyclodextrins, may be hydrolyzed by lipases without significant changes in the enzyme kinetic properties and stability [44].

Although ACN decreases the lipase stability in aqueous systems, CCL suspended in nearly dry ACN (3% v/v) was found to be more stable than in pure water (Table 1). Several reasons have

been given to justify the stability enhancement in nearly anhydrous organic media [24,25]. In these conditions, the lipase is not solubilized in the medium, but it is suspended and dispersed on it. The conformational mobility of insoluble protein is seriously restricted. Also, water activity is very low. Thus, its action in the deamidation of asparagine residues, formation of incorrect (scrambled) structures due to disulfide interchange [45], hydrolysis of peptide bonds and destruction of cystine residues [46] responsible for irreversible enzyme inactivation, is minimized. The absence of the necessary amount of water for these processes, and to dissolve the protein in the medium, is considered to be the main cause of the stability increment in nearly anhydrous organic media.

The suspended protein in the organic medium is solvated by a certain number of water molecules. In biocatalysis it is required that water forms hydrogen linkages with functional groups of the protein [34,35]. In the case of nearly anhydrous polar solvents, a great deal of the enzymes are not active, due to its low hydration [47]. Table 1 shows the high resistance of CCL during its storage in nearly anhydrous ACN (0–5% v/v buffer). The absence of activity in reactions with CCL in nearly anhydrous ACN [26] has been attributed to the presence of suspended protein, in a non active form. Our results showed that suspended protein in ACN may recover 100% of its activity when it is dried to total ACN elimination, and then solubilized in water (see Methods). That is, the protein in the inactive conformation in ACN is more stable and, inactivation is reversible.

The effect of water content (A_w) is not only relevant in CCL activity, but also in its stability. ACN with 3% v/v of aqueous buffer is a better storage medium for CCL than pure water, however the enzyme is not active. Additives (i.e., ethylene glycol) that can mimic the action of water [48] may be used to work in media where lipase has the highest stability. Also, the lipase incubated in anhydrous ACN is able to recover its activity, if it is dissolved again in aqueous buffer, i.e., the inactivation is reversible.

High contents of water in ACN ($>3\%$ v/v) diminish its stability due to the solubilization of suspended protein. It has been described that protein denaturation is favored when the enzyme is solubilized. The kinetic and spectroscopic data of the present work show that these media are less favorable than pure water because the main effect on the activity decay is due to small changes in orientation of some protein groups – not detected by fluorescence spectroscopy – when it is solubilized in absence of appropriate water solvation. The reduction of transition temperatures of proteins in water-miscible organic solvents such as lower alcohols have been described [49]. The protein unfolding and its folding into a new, thermodynamically stable, but inactive in anhydrous media, structure has been described like the thermoinactivation mechanism of enzymes [50]. This hypothesis is in accord with the thermoinactivation studies of enzymes in organic media–water systems, that have shown an activation energy much higher than that corresponding to a covalent process [51], and typical for conformational processes.

These results support that the principal effect of an organic solvent in the stability and/or activity is not due to its interaction with functional groups of the enzyme molecule, but with water used in protein hydration [26], reducing enzyme mobility and chemical denaturing processes of water. But also, it shows two kinds of conformational changes for enzyme denaturation in polar cosolvents–water systems: an initial change favored by a low protein solvation, when protein is solubilized, and other unfavored by low A_w systems, related to Trp emission.

Fig. 2 and Table 1 show that also some protein hydration is necessary for optimal stability in nearly anhydrous media. The lower aqueous content obtained for optimal stability of CCL in n-heptane (Fig. 2) compared with ACN (Table 1), may be justified considering the higher interaction of ACN with water, and the type of agitation, more effective in ACN. A more effective agitation decreases enzyme hydration, because enzyme molecules cannot share the water present [52].

CCL easily loses the water molecules of its solvation/hydration shell in polar media [26].

High molecular aggregation of protein with high amount of water shared for enzyme molecules, and partial solubilization of lipase could explain a change of texture in n-heptane: the lipase suspension adopts a paste form after certain length of time.

Half inactivation time of CCL in heptane–water systems tends to the value in the aqueous medium. This reflects not only the total absence of direct protein–heptane interaction, but also the low solvent–water interaction. At water contents higher than 3% v/v, protein is solubilized and the aqueous environment of enzyme presents physico-chemical properties similar to that of a pure aqueous solution. Similar physico-chemical properties of solvation shell of protein, in presence and absence of n-heptane, may explain the monophasic curves for n-heptane–water systems in Fig. 3, in contrast with biphasic inactivation curves obtained in Fig. 1 with ACN.

The influence of the solvent nature was studied using mixtures of solvents. These mixtures were used in place of pure solvents to enhance the effect of Lp, minimizing the effect of other solvents properties in each two solvent mixture. The existence of two intervals in Fig. 3 shows that lipase stability at constant water content, depends on the molecular nature of solvent and the agitation effectiveness (D/L_{MB}). Solvent properties of different Lp, and D/L_{MB} value are distinct in the two intervals. According to this figure, the stability is favored when Lp decreases, but other molecular properties of the solvent also apply. The comparison between the two intervals showed that although Lp is not the sole solvent property that determines enzyme stability, it may be considered as the main factor among solvent properties. Water content and agitation type (D/L_{MB}) are also key parameters in CCL stability. The appropriate water content must be found to keep the active conformation, but not excessive, otherwise it favors enzyme solubilization and denaturation by chemical action and protein unfolding. The high stability in polar media, i.e., in ACN, is due to the high water–solvent interaction. In these conditions, there is no free water, and its negative effect on the enzyme is lower. At constant water content, when the Lp increased the water activity also did, because the water–solvent interaction was reduced. Isotherms of adsorption of water for proteins have shown that in polar solvents, the water adsorbed by the protein, does not exceed a given value, but in apolar media it reaches the maximal value [53]. In an apolar medium, more water is free to dissolve the protein (decrease of protein rigidity) and to act in chemical processes of enzyme denaturation. Thus, when Lp is enhanced at constant water content, two negative effects are favored: protein hydration/solubilization, and chemical denaturing processes.

Fig. 3 shows again that the absence of CCL activity in ACN, described by Zaks and Klibanov [26], is not due to its irreversible inactivation.

ACN with 1% v/v of water content resulted the best solvent in Fig. 3, but it has been already discussed that polar media with high water content ($>3\%$) solubilize the protein and stabilize partially inactivated conformations.

The stabilization by water structuration in solvents that does not interact with water, like n-heptane, may be carried out with the addition of PEG (Fig. 4). Thus CCL was found to be nearly 3 times more stable with 1/1 w/w PEG, in presence of 1% v/v water. PEG is a depressor of water activity, reducing protein hydration, and thus its partial solubilization and chemical action of water in protein degradation. Thus, similar stability presented CCL with PEG and 1% v/v of water, than protein without additive and half content of water (insert, Fig. 4). Polyphenol oxidase in chloroform presents similar stabilization, and although its effect does not depend on the PEG concentration, this is more effective with PEG of high MW [51]. The necessity of water structuration to increase the enzyme stability in apolar media, supports the hypothesis of its essential role in the irreversible inactivation of enzymes in organic media. PEG has been used in other strategies to improve the activity/stability of solubilized enzymes in polar and apolar media [54]. The covalent linkage of

PEG molecules to proteins, makes the enzymes able to act solubilized in organic media. But these processes require higher water amounts than reactions with native proteins. Thus, the enzyme molecules are surrounded by highly hydrated PEG molecules [55]. The stability of modified enzymes is usually higher than that of the solubilized native enzyme. It is due to the ability of PEG to retain water for the protein solvation in polar media. At the same time, chemical processes of denaturation are minimized when they are used in apolar media, due to the high degree of structuration of water. The positive effect of PEG addition in the stability of lipases A and B in ACN, at relatively low protein/water ratios, is also due to the increase of water structuration (Table 3).

There are other chemical modifications of enzymes which produce stabilization. Modification of CCL with dithiothreitol increased the operational stability of CCL in apolar media, without significant change in its activity. However, the positive effect of reduction of disulphide bridges has not been clarified [56].

Reactor design is also a key factor for enzyme stability. From Table 2 it can be seen that a small trituration effect of CCL could take place. But the decrease of stability when the size of magnetic bar increases, is essentially due to its effect in the lipase aggregation. The aggregation of suspended lipase has two effects. The associated molecules of protein protect themselves from conformational changes. Also in the aggregated state, the enzyme molecules share water molecules. In polar media with low water content – ACN in Table 2 –, the aggregated lipase may obtain its optimal hydration for its stability, sharing water among all lipase molecules. This is consistent with the higher positive effect of the aggregation with the lower buffer content. An increase of CCL activity, when the hydration degree of aggregated lipase is enhanced, has been described [52] for the production of heptyl oleate. In this case, a distinct hydration degree was obtained with a change in the agitation speed of system, at constant D/L_{ML}. Similarly, the effect is more important in systems of lower A_w.

In n-heptane with low and constant water content (1% v/v), the ability of water to act in chemical processes of enzyme denaturation diminishes when protein/water ratio increases; at the same time increasing the protein rigidity. This positive effect of a high enzyme concentration may be more important in a process on industrial scale.

Values of $t_{50\%}$ for lipases A and B in ACN also increased when higher enzyme amount was used, due to the aggregation effect and the increase of protein/water ratio. The effect of A_w depressors was found to be higher for purified lipases than for crude CCL containing 35–40% of lactose. In general, polyols with higher depressor character of A_w, produced higher stabilization effects in purified lipases (Table 3), but also the influence of the molecular properties of additives. Thus, additive effects are in the order: high MW linear polyol < cyclic mono- and disaccharides < low MW linear polyol. The small and less bulky additives are more effective stabilizers. Lipases A and B have the same stability when the negative effect of water molecules is seriously diminished with additives (about 43–46 h in Table 3). From the results in Table 3, it can be considered that the high stability of crude CCL in ACN is mainly due to the presence of polyols (35–40% of lactose) in the commercial lipase powder, and the aggregation/protection effect of impurities. Different stability found for isospecies A and B in absence of additives, may be due to the different purification degree, which is higher for lipase B, and also the distinct water content of the incubation media (1% and 1.5% v/v, respectively). Thus, the stability order of lipase from *C. cylindracea* is the same as the order of purification degree: CCL (11% w/w) > CCL A (63% w/w) ≫ CCL B (97% w/w). Also, experimental curves of lipase A inactivation – a single isoenzyme – can be explained by an single exponential decay. Thus $t_{50\%}$ represents its half life. But the experimental inactivation of lipase B follows a triple exponential decay, in accord with its composition – four isoenzymes with the same N-terminal sequence.

This study showed that factors like agitation types and enzyme amount are relevant in the CCL

stability, and it must be considered beside A_w and the solvent properties, in order to design a given process. Negative effects of the purification degree may be compensated using appropriate additives or conventional stabilization/immobilization methods.

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