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Effect of the Pretreatment of Lipase with Organic Solvents on its Conformation and Activity in Reverse Micelles

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Abstract The activity and conformation of *Chromobacterium viscosum* lipase-pretreated with various organic solvents were investigated. The pretreatment of lipase led to a substantial increase of enzyme activity in AOT (sodium bis [2-ethyl -1-hexyl] sulfosuccinate)/isooctane/water reverse micelles. Among the organic solvents used, *n*-hexane was found to be most effective. It was observed that higher hexane content with shorter agitation time and vice versa had almost the same effect on the initial activity of lipase. The kinetic study showed that the Michaelis constant (K_m) and the substrate adsorption equilibrium constant (K_{ad}) were reduced by the pretreatment of lipase with hexane, whereas the change in the maximum reaction rate (V_{max}) was insignificant. The two spectroscopic techniques (Fluorescence spectra of lipase encapsulated in RMs and Fourier transform infrared [FTIR] spectra of lipase powders) were performed to detect possible conformational changes in the enzyme caused by the pretreatment. A correlation between the maximum fluorescence intensity and the activity of treated lipase was found as a function of agitation time. The FTIR spectrum of lipase showed a new shape peak corresponding to 1,500 cm^{-1} as a result of pretreatment with organic solvents.

Keywords Lipases · Pretreatment · Hexane · Hydrolysis · FTIR · Fluorescence

Introduction

Reverse micelles (RMs) are generally described as nanometer-sized water droplets dispersed in an organic solvent with the aid of a surfactant monolayer, forming a thermodynamically

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stable and optically transparent solution. Biomolecules such as enzymes are solubilized inside the micellar core, shielded from the bulk organic phase by the layer of surfactant, and hence their enzymatic activity and stability remain unharmed [1, 2]. Water-insoluble substrate can also be easily dissolved in nonpolar phase. In addition, the macroscopic homogeneity transparency of this system permits the use of continuous spectrophotometric techniques for the investigation of enzymes conformation. In view of these advantageous features, reverse micellar systems have been extensively used to investigate enzymatic reactions containing water-insoluble substrates or products. Many surfactants and solvents can be used to form RMs [3]. The AOT (sodium bis [2-ethyl -1-hexyl] sulfosuccinate)/ isooctane system is used as one of the most suitable systems [4–6], as the RMs formed by this surfactant are very stable over a wide range of concentrations in the absence of cosurfactants. The AOT RMs are well defined with respect to size, shape, and aggregation number. The solvent used to form RMs can largely influence the enzyme activity [7, 8]. Han and Rhee [8] have compared the activity of lipases in different RMs prepared with various organic solvents, and isooctane gave the best activity. Isooctane has the structure similar to the tail structure of AOT and thus has the best penetration into AOT tails [8].

Lipases (triacylglycerol hydrolases, EC 3.1.1.3) are a special class of enzyme that is only activated at an oil–water interface [9, 10]. Because RMs provide the large interfacial area to activate the lipase, they are particularly attractive for lipase-catalyzed reactions [2, 3, 6, 11–15]. The active site of *Chromobacterium viscosum* lipase (CVL) is located in the hydrophobic amino acids region and is generally buried under a lid or flap, making the active site inaccessible to the substrate in the so-called closed conformation [16]. The movement of the lid is necessary for opening the active site to make it accessible to substrate [16–18]. If the active site can be kept open in advance, lipase is expected to have a higher activity in RMs than that of untreated lipase, which is usually in closed form. The pretreatment of enzymes with polar organic solvents has been reported to be very effective to open the lid covering active sites [19–22], and the catalytic activity and enantioselectivity of enzymes are enhanced significantly. However, very little is known about the effect of the pretreatment of CVL with nonpolar organic (hydrophobic) solvents on its enzymatic performance in RMs. The spectroscopic analysis can also be used to evaluate the direct structural information of the treated lipase.

In the present study, the effect of the (pre)treatment of CVL with various hydrophobic solvents on its activity in RMs and a possible conformational change is described. This study is also addressed to the investigation of lipase structure by fluorescence and Fourier-transform infrared (FTIR) analysis. In addition, the kinetic parameters of treated and untreated lipases is also compared to better understand the activity improvement.

Materials and Methods

Enzyme and Reagents

Lipase from *C. viscosum* (3,900 units/mg solid) was kindly provided by Asahi Chemical Industry Co. Ltd. (Tokyo, Japan) and used without further purification. AOT (sodium bis [2-ethyl-1-hexyl] sulfosuccinate), cyclohexane, *n*-hexane, *n*-heptane, and *n*-octane were purchased from Wako Pure Chemical Industries, Co (Osaka, Japan). Highly refined olive oil was also obtained from Wako Pure Chemical Industries, Co. with a saponification value of 192. All chemicals used were of analytical grade and used as received.

Pretreatment of Lipase

The enzyme (20 mg) was dissolved in predetermined amounts of Brinton buffer (pH 7.2) at 4°C with gentle stirring for 30 min. Different volume percentages of organic solvent were added up to a final volume of 10 ml, followed by agitating at 500 rpm in an incubator at 4°C. After freezing the solution at −80°C for approximately 12 h, the sample was dried under a freeze dryer (EYALA, FDU-506) at a pressure of approximately 8 Pa and a condensed temperature of −50°C for 24 h. Treated lipase powder was stored at −20°C until its use.

Lipase Microencapsulation Methods in Reverse Micelles

Injection method This method is mostly used to microencapsulate enzymes because of its simplicity. Powder lipases (treated and untreated) were first dissolved in a buffer solution and then an appropriate amount of lipase solution was injected into the reverse micellar solution (the amount of water determined according to w_o). The mixture was agitated until a clear and optically transparent solution was obtained.

Dissolution method The powder lipases were directly added to reverse micellar solution and agitated at 500 rpm for 15 min; the solution was then centrifuged at 1,000 rpm and the supernatant used as the lipase-containing micellar solution. The lipase content in the supernatant was determined by the measurement of absorbance at 278 nm using a Shimadzu spectrophotometer (BioSpec-1600). In this study, treated and untreated lipases were encapsulated in RMs by the dissolution method unless otherwise mentioned.

Preparation of Reverse Micelles (RMs)

Dissolving AOT in isooctane with a limited amount of water formed reverse micellar solution. Reverse micelles containing lipase (2 mg dm^{-3}) were prepared by adding an appropriate amount of reverse micellar solution in which the freeze-dried lipase had been dissolved in advance. Water–AOT molar ratio (w_0) was adjusted by adding a desired amount of buffer solution. The reverse micelles volume was also adjusted by adding a desired amount of AOT/isooctane solution. A magnetic stirrer was used to agitate the mixture until a clear and optically transparent solution was obtained. The micellar solution containing lipase was placed in an incubator at 25°C until the hydrolysis reaction was initiated.

Determination of Lipase Activity

The activity of lipase was obtained using the same procedures as reported in the literature [23, 24]. Briefly, lipase activity was defined as the initial reaction rate, V (mol fatty acids dm^{-3} s^{-1}). The reaction was commenced by adding an appropriate amount of olive oil into a preincubated micellar solution containing lipase. The reaction mixture was agitated with a magnetic stirrer, at 500 rpm in an incubator for exactly 20 min, as it was found that the production of free fatty acid was linearly dependent on time (data not shown) in this range. The resultant fatty acid was analyzed by the Lowry technique [25]. In this study, all of the concentration terms were based on the total volume of the reverse micellar system unless otherwise specified.

Fluorescence Measurement

Steady-state fluorescence emission spectra of treated and untreated lipases encapsulated in RMs were measured with a Hitachi F-3010 fluorescence spectrophotometer. The excitation wavelength was 280 nm. The emission wavelengths varied from 300 to 400 nm in 1-nm increments. The slit widths of the excitation and the emission were 5 nm. Spectra were uncorrected for instrument sensitivity, but the emission of blank RMs (without lipase) was subtracted.

FTIR Study

The FTIR measurements were performed at absorbance mode using Horiba FT-210 Fourier transformed infrared spectrometer with a baseline horizontal attenuated total reflectance (HATR) cell. The data were recorded by a Horiba Spectradesk. FTIR scans were carried out on the lipase powders. A total of 256 scans at 2 cm^{-1} resolution were averaged. FTIR spectra were recorded in a 400–4,000 cm^{-1} range. An empty cell was used as the blank cell. All the spectra were taken at room temperature.

Results and Discussion

Effect of Hydrophobic Solvents on Lipase Pretreatment

The organic solvents with different log *P* values (widely used for measuring hydrophobicity) have been used for the pretreatment of lipase. Figure 1 shows the activity of treated lipases as a function of agitation time. The log *P* values of cyclohexane, hexane, heptane, and octane are 3.2, 3.5, 4.0, and 4.5, respectively [26]. Although a notable difference in the maximum activity is not found among the lipases treated with various hydrophobic solvents, optimized agitation time corresponding to the maximum lipase activity decreases with the increase in solvent log *P* value at a constant volume ratio of solvent to water. This is also supported by Fig. 2, where it is found that the optimized agitation time decreases with the increase of hexane content, i.e., with the increase of hydrophobicity in the pretreatment media. The activity data corresponding to agitation times less than 10 min is not stable.

Results in Figs. 1 and 2 show that the lipase activation depends on agitation time (the time of lipase treatment with organic solvent). The pretreatment with organic solvent is expected to result in a conformation change of the enzyme, exposing a large hydrophobic surface that also includes the active sites [21, 27–29]. With the increase in agitation time, the conformation of lipase changes from a less hydrophobic closed form to a more hydrophobic open form. This may enhance the affinity of lipase for the hydrophobic substrate. Therefore, the optimum agitation time corresponds to a state in which the affinity between treated lipase and substrate reaches a maximum. As a consequence, the active site of the treated lipase can easily contract with hydrophobic substrates, and the lipase activity is improved. At higher agitation times, the decrease in lipase activity could be attributed to the lipase denaturation as a result of the long-term interaction with organic solvents. On the other hand, the addition of hydrophobic solvents such as hexane in water–solvent mixture enhances the interfacial area beneficial for lipase activation [30, 31]. This large interfacial area presumably allows the lipase to solubilize itself smoothly with a higher concentration, and accordingly conformational change of lipase can be effected more rapidly. Because

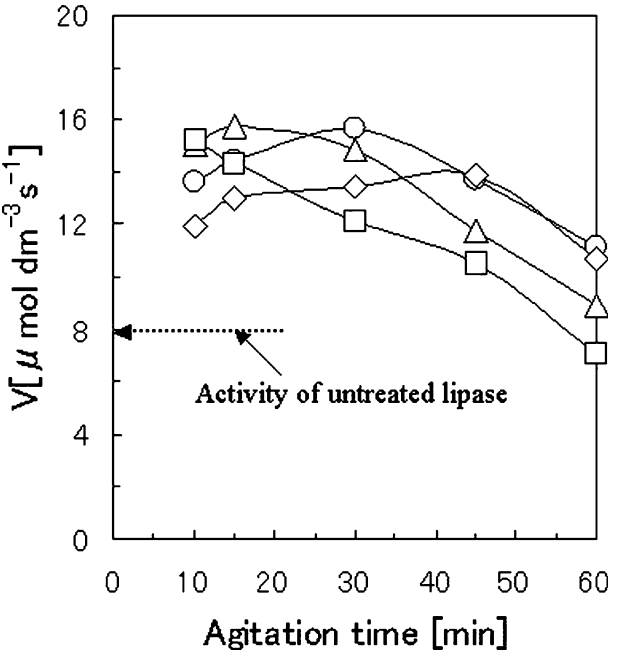

Fig. 1 Hydrolytic activity of lipase pretreated with various hydrophobic solvents in RMs: (◇) cyclohexane, (<) *n*-hexane, (ñ) *n*-heptane, (E) *n*-octane. Pretreatment conditions: solvent/water (*v*:*v*) =1:1, agitation temperature = 4°C, lyophilization time = 24 h. Activity assay conditions: C_{lipase}= 2 mg/dm^{-3}, C_{AOT} = 50 mM, $C_{olive\ oil}$ = 55 mM, w_o (molar ratio of water to AOT) = 10, buffer pH = 8, reaction temperature = 25°C

both the interfacial area and agitation time facilitate the pretreatment of lipase, higher interfacial areas of the activated media with a shorter agitation time or vice versa may have the same effect on the change in lipase conformation and activity.

Among the organic solvents used as the pretreatment media, hexane is found to be most effective in enhancing lipase activity. The results in Fig. 2 suggest that the pretreatment

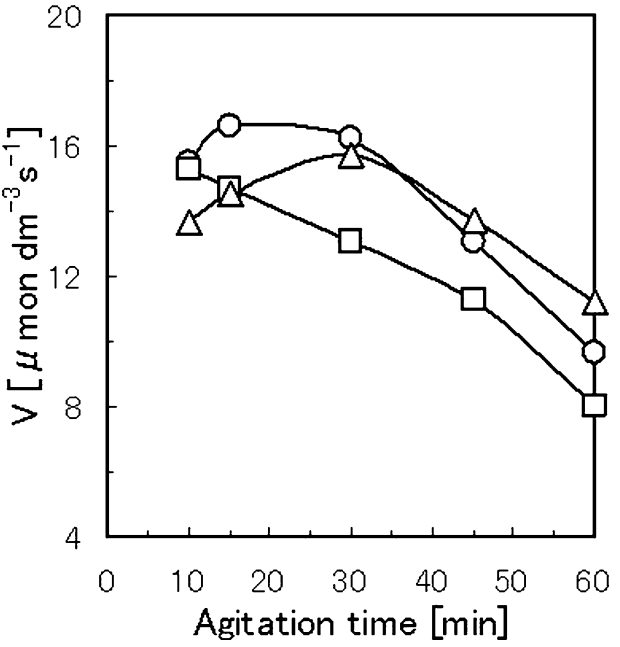

Fig. 2 Effect of *n*-hexane content (*v*/*v*) during pretreatment: (E) 3:1 (solvent/water), (<) 2:1, (ñ) 1:1. Activity assay conditions are the same as those in Fig. 1

medium with a volume ratio of hexane to water of 2:1 is the most effective to activate the lipase, and the maximum activity of lipase treated at this condition is 1.95 times higher than that of untreated lipase. Therefore, hexane with a volume ratio to water of 2:1 is selected as the pretreatment medium in subsequent experiments unless otherwise stated.

Reaction Kinetics

The performances of treated and untreated lipases in terms of the initial rate of the hydrolysis of olive oil into fatty acids and glycerol are compared. A kinetic model (Eq. 1) [23] based on the adsorption equilibrium of the substrate between the organic and micellar phase is used to better understand the lipase activation by the pretreatment with hexane.

$$v = \frac{V_{\max}[S_T]}{K_m(1 + K_{ad}[C_S]) + [S_T]} \tag{1}$$

where,

V Reaction rate;
K_m Michaelis constant
K_{ad} Adsorption equilibrium constant, where $S_{ad} = K_{ad}\,[C_s]$
C_S Surfactant concentration participated in micellar formation
S_{ad} Total substrate adsorbed on the micellar surfactant surface
S_T Total substrate (olive oil) concentration

The values of various kinetic parameters are determined using Lineweaver–Burk plots [23] and listed in Table 1. For convenience of comparison, the kinetic parameters for the untreated lipase are also listed. It is seen that the Michaelis constant (K_m) and substrate adsorption equilibrium constant (K_{ad}) are reduced by the pretreatment, whereas maximum reaction rate (V_{max}) remained unaltered. As the K_m value reflects the affinity between enzyme and substrate, the decrease in K_m indicates that the binding affinity between enzyme and substrate increases [32], possibly as a result of the change in lipase conformation during the pretreatment. The K_{ad} value reflects two factors [33]: the adsorption of substrate on the surfactant tails, and the electrostatic and hydrophobic interactions between lipase and AOT molecules. Because lipase molecules exist within the micellar core, the pretreatment of lipase could not affect the substrate adsorption on the tails of AOT. Therefore, the significant decrease in K_{ad} value suggests that the electrostatic and hydrophobic interactions between lipase and AOT molecules are reduced.

Fluorescence Study

The effect of the pretreatment of lipase with hexane on its conformation is investigated by steady-state fluorescence spectroscopy, monitoring the intrinsic emission of the tryptophan

Table 1 Comparison of kinetics parameters for treated and untreated lipase in AOT RMs.

Kinetic parameters	Hexane-treated lipase	Native lipase [x23]
V_{max} (μmol dm^{-3} s^{-1})	29.8	27.0
K_m (mol dm^{-3})	0.032	0.083
K_{ad} (mol^{-1} dm^3)	5.7	16.2

residues. These residues of enzymes serve as intrinsic fluorescence reporters for the molecular structure and dynamics of the respective enzymes. The three dimensional crystal structure of CVL has been extensively investigated [34] and found to be identical to a lipase from *Pseudomona glumae* [35]. They have reported that the amino acids sequence of CVL consists of 319 residues, including three tryptophans (Trp; 30, 209, 283), among them Trp 209 and Trp 283 are closely located to the lipase active site (catalytic triad Ser 87, His 285, and Asp 263). They are almost completely buried in the interior (hydrophobic core) of the protein and therefore are hardly accessible to substrate molecules.

Figure 3 shows the fluorescence intensity of treated and untreated lipases encapsulated in AOT reverse micelles by two different methods: (1) injection and (2) dissolution. The intensity of treated lipase encapsulated in RMs by dissolution method is increased significantly compared with that of untreated lipase. However, when lipase was encapsulated by injection method, the intensity of treated and untreated lipases is almost the same. This result suggests that the pretreatment with hexane renders the lipase surface more hydrophobic through the exposure of Trp residues, whereas the previous exposed hydrophilic residues become buried inside the enzyme [18, 36]. The exposed hydrophobic residues to the lipase surface enhance the hydrophobic interactions, improving the binding between lipase active site and hydrophobic substrate [21, 28, 37]. The lower intensity of treated lipase during the injection method indicates that this conformational change is reversible when dissolved in water. In fact, when treated lipase is dissolved in water, it may return to its native conformation, and its Trp residues are further hidden in the lipase core [38]. On the other hand, when treated lipase is encapsulated in RMs, the change in lipase conformation accompanied by the pretreatment is retained because of the insufficient water (less than 1% water, *v*/*v*) and the hydrophobic state of water bound to the micellar interface [3, 39].

Figure 4 shows that the maximum fluorescence intensity increases with the increase of agitation time up to a maximum value and then begins to decrease. The similar tendency is seen in case of the variation of the activity of hexane treated lipase with agitation time shown in Fig. 2. Combining the results in Figs. 2 and 4, it appears that a correlation may

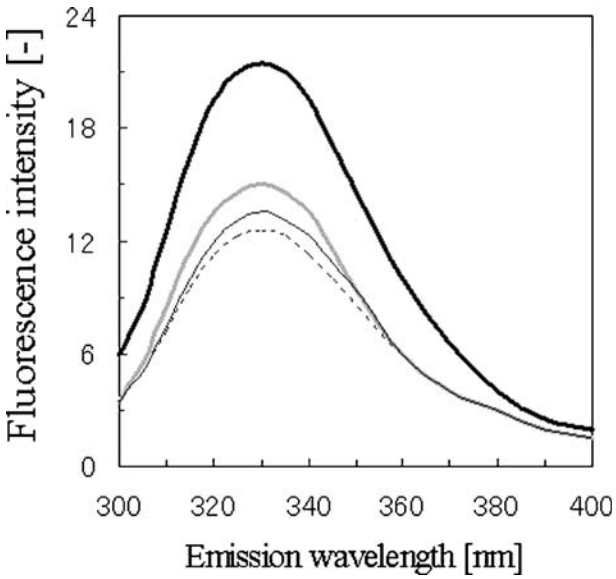

Fig. 3 Fluorescence emission spectra of treated and untreated lipase being encapsulated in RMs by two methods: (▬) treated lipase with dissolution method, (▬) untreated lipase with dissolution method, (—) untreated lipase with injection method, (····) treated lipase with injection method. Pretreatment conditions: *n*-hexane/water =2:1, agitation time = 30 min, lyophilization time = 24 h. Experimental conditions: C_{lipase}=10 mg dm^{-3}, C_{AOT}=50 mM, buffer pH =8, w_o=10

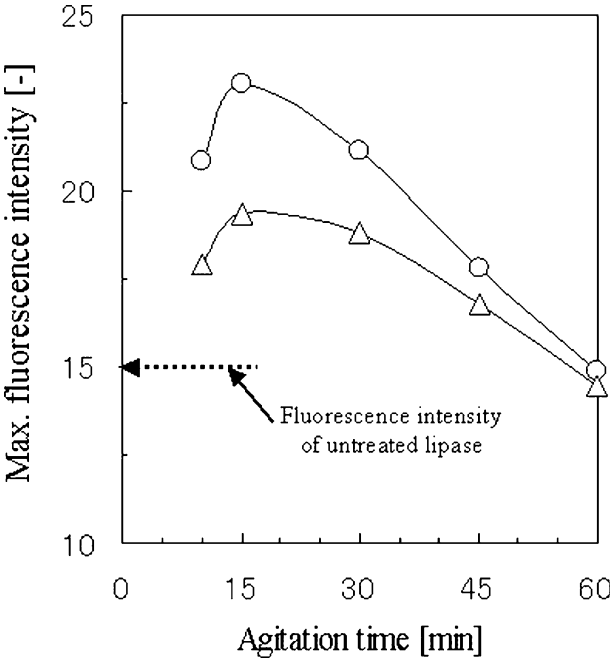

Fig. 4 Variation of the maximum fluorescence intensity with the agitation time at different *n*-hexane contents (hexane/water = *v*/*v*): (<) 2:1, (ñ)1:1. Other experimental conditions are the same as those in Fig. 3

possibly exist between the fluorescence intensities and the activities of treated lipase. It is also notable that the tryptophans fluorescence intensity of treated lipase increases with the increase of hexane content in the pretreatment media. This indicates that the change in lipase conformation is strongly dependent on the interfacial area in the pretreatment media created with the addition of hydrophobic solvent.

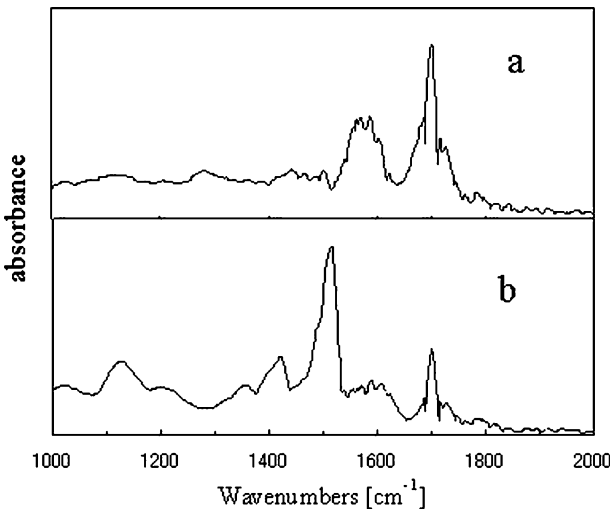

Fig. 5 FTIR spectrum of lipase. (a) Untreated *C. viscosum* lipase, (b) treated *C. viscosum* lipase

FTIR Study

FTIR spectra of treated and untreated lipases are obtained to detect possible conformational changes in the enzyme molecule. Figure 5 shows the FTIR spectra of powder lipase in the region of 1,000 to 2,000 cm^{-1}. In contrast to the untreated lipase, a clear-cut difference is observed in FTIR spectra of treated lipase. No considerable peak at about 1,500 cm^{-1} is observed in case of untreated lipase (Fig. 5a), whereas a new distinct peak at 1,500 cm^{-1} appeared in the case of treated CVL (Fig. 5b), with a peak at 1,700 cm^{-1}. Murray et al. [40] have reported that FTIR peaks at wave numbers approximately 1,500 and 1,700 cm^{-1} represent the exposed amino group and carboxylic group, respectively.

As noted previously, the active site of lipases, in general, contains a catalytic triad consisting of serine, histidine, and asparagine [35]. The catalytic triad is generally buried by a loop helix, making the active site inaccessible to the substrate. It opens during the interfacial activation to facilitate substrate access. No peak appeared at 1,500 cm^{-1} in the case of the untreated lipase, which suggests that amino group of enzyme (at lid helix) is hidden in the core. In contrast, appearance of a new peak at 1,500 cm^{-1} indicates that lipase is being treated in such a way, which may expose reactive amino groups, with active site in open conformation. FTIR results further suggest that the pretreatment with organic solvents converts the closed form of CVL to the open form, thus imparting a high activity to the treated lipase in RMs.

Conclusions

The pretreatment of lipase with organic solvents was a simple and effective technique to improve its catalytic activity in AOT RMs toward hydrolysis reactions. This pretreatment of enzymes has provided important insights in enzyme structures such as the amino acids involved in the active site of enzymes, and this may be useful in some applied conversions catalyzed by lipases in organic media. The kinetic results indicated that the pretreatment led to improve the affinity between substrate and lipase and reduce the interactions between them. The pretreatment of lipase with organic solvents rendered the lipase surface more hydrophobic, facilitating the interaction with hydrophobic substrate. The conformational change in the enzyme produced by the pretreatment was proven with the FTIR analysis.

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