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Enhanced activity and stability of *Chromobacterium viscosum* lipase in AOT reverse micellar systems by pretreatment with acetone

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Abstract

The activation and stabilization of *Chromobacterium viscosum* lipase (glycerol-ester hydrolase, EC 3.1.1.3) by a simple pretreatment with acetone was studied in AOT/water/isooctane reverse micellar systems, using the hydrolysis of olive oil as a model reaction. The maximum activity of acetone treated lipase was found to be 160% higher than that of native lipase. These observations were rationalized by the assumption based on the lipase conformational changes accompanied by the enhancement of lipase surface with hydrophobic amino acids. The dependence of catalytic activity on the various parameters relevant to the pretreatment procedure such as acetone content, the pH of water–acetone solution, agitation time of the lipase in water–acetone solution and freeze-drying time were investigated. The change in lipase conformation due to the treatment with acetone was confirmed by typtophyl fluorescence spectroscopy as well as lipase microencapsulation methods in reverse micelles. The acetone treated lipase entrapped in AOT reverse micelles was very stable, retaining over 83% of its initial activity after 40 days, whereas the half-life of native lipase was 38 days.

Keywords: Hydrolysis; Acetone pretreatment; Hydrophobic amino acids; Reverse micelles; Lipase activity and stability

1. Introduction

The production of fatty acids through the hydrolysis of oils and fats has emerged as a major area of biotechnology research and development. Conventional methods of hydrolysis operate at high temperature and pressure, and these drastic conditions demand high-energy consumption as well as the formation of undesirable byproducts. But enzyme-catalyzed hydrolysis is carried out under mild conditions, reducing the secondary reaction to a very low level of pronounced enzyme selectivity. Although enzymes are typically used in the aqueous media, there are several advantages of enzymatic hydrolysis in organic solvents, such as high substrate solubility, less microbial contamination and less product inhibition [1–3]. Catalytically, lipases act only at or near the interface [4,5]. Compared with other interfacial media, reverse micelles provide a larger interfacial area that promotes contact between enzyme and substrate [6]. In reverse micelles, enzymes are solubilized in a micellar core, hence protected from the detrimental effect of organic solvents by the surfactant layer [7]. The anionic double-tailed surfactant, AOT [sodium bis(2-ethyl-1-hexyl) sulfosuccinate], is frequently used in micellar technologies. However, activities of enzymes entrapped in AOT reverse micelles are inhibited by strong interactions with surfactant molecules [8–11] and the distinctive properties of micellar water [12].

To overcome these problems, an improvement of enzyme activity has been reported using a newly synthesized surfactant [13] or a modified surfactant (AOT) [14]. But such modification and synthesis of surfactants are laborious and time-consuming. Many researchers modify reverse micellar systems by using additives to enhance the lipase activity [15–19]. Another approach is to chemically modify the

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Nomenclature

AOT	sodium bis(2-ethyl-1-hexyl) sulfosuccinate
C_{AOT}	AOT concentration (mol dm^{-3})
C_{lipase}	lipase concentration (mg dm^{-3})
C_{NaCl}	NaCl concentration in buffer (mol dm^{-3})
$C_{olive\ oil}$	olive oil concentration (mol dm^{-3})
C. viscosum	*Chromobacterium viscosum*
w_0	molar ratio of water to AOT in the micellar system

enzyme surface [20–22]. But specific hydrolytic activities of lipase are significantly reduced by the chemical modification [20]. No reports are available about the enhancement of the lipase activity entrapped in AOT reverse micelles by pretreatment with organic solvents that may cause the changes in the lipase conformation.

The enhancement of catalytic activity of enzymes in organic media by pretreatment with organic solvents has progressed gradually [23,24]. The pretreatment of enzymes with organic solvents is relatively simple and less time-consuming than chemical modifications. The protein surface is balanced with hydrophilic–hydrophobic residues, and thus the enzyme surface is primarily responsible for the interaction with surrounding environments. Enzymes are generally structured to keep a majority of hydrophilic amino acids on the surface and hydrophobic amino acids in the core. The active site of lipolytic enzyme (e.g. *C. viscosum* lipase) locates in hydrophobic amino acid regions and combines with hydrophobic substrates (e.g. olive oil) at the micellar interface through hydrophobic interaction. Therefore, the affinity (the energy of binding between the enzyme and hydrophobic substrate) may be improved by increasing the exposure of hydrophobic amino acids on the enzyme surface. It has been reported that pretreatment with organic solvents such as acetone converts the lipase conformation from closed form (active site is covered by a lid) to open form (active site is uncovered) through the exposure of hydrophobic amino acids to the lipase surface [23]. A similar phenomenon has previously been reported in the literature [25,26]. It is reasonable to assume that pretreatment may lead to a change in lipase conformation to some extent, which improves the interaction between lipase active site and substrate. In addition, increased hydrophobic amino acids on the enzyme surface may contribute to enzyme activation and stabilization by making the lipase environmentally compatible inside the hydrophilic water pool that usually inhibits the access of the lipase active site to the micellar interface.

The objective of this study is to enhance the activity and stability of lipase entrapped in AOT reverse micelles by pretreatment with acetone. Another aim is to investigate the optimum pretreatment conditions. The choice of acetone as a modifying reagent is based on its water miscibility, allowing the modification to be performed in water. It is evident that the conformational change in lipase during the pretreatment is retained after freeze-drying.

2. Materials and methods

2.1. Materials

Purified *C. viscosum* lipase (glycerol-ester hydrolase, EC 3.1.1.3) was provided by Ashahi Chemical Industry Co. Ltd. It is a mixture of lipase A with a molecular weight of 120 000 (pI = 3.7) and lipase B with a molecular weight of 30 000 (pI = 7.3). Lipase B is the main portion of the mixture and its content by weight is over 80% [27]. AOT [sodium bis(2-ethyl-1-hexyl) sulfosuccinate], isooctane, benzene, pyridine, olive oil, copper (II) acetate and acetone were purchased from Wako Pure Chemical Industries Co. The average molecular weight of the olive oil was 875 g mol^{-1} and density was 0.91 g cm^{-3}. All chemicals were analytical grade and used without further purification.

2.2. Pretreatment of lipase with acetone

The pretreatment was performed by adding 1 mg/ml of lipase into a solution of 50% (volume) cold acetone in Brinton buffer, followed by agitating for a desired time by a magnetic stirrer at 500 rpm in an incubator at 4 °C. After freezing the solution at −80 °C for about 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. Lyophilized lipase powders were stored in a freezer at −20 °C.

2.3. Lipase microencapsulation methods in reverse micelles

2.3.1. Injection method

This method is mostly used to microencapsulate enzymes due to its simplicity. The powder lipase was 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_0). The mixture was stirred until a clear and optically transparent solution was obtained.

2.3.2. Dissolution method

The treated lipase (freeze-dried powder) was directly added to the reverse micellar solution and agitated by a magnetic stirrer at 500 rpm in an incubator at 25 °C for 15 min. After centrifuging (HIMAC centrifuge, Hitachi, model CR15) at 1000 rpm for 5 min, the upper phase was separated carefully. The lipase content in the upper phase was determined by UV method. The absorbance was detected at 278 nm by double beam Spectrophotometer and the lipase concentration was calculated from the standard curve. In this work, treated and

untreated lipases were dissolved by the dissolution method unless otherwise specified.

2.4. Preparation of reverse micelles

Dissolving AOT in isooctane with a limited amount of water formed reverse micelles. 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 the desired amount of buffer solution. The reverse micelles volume was also adjusted by adding the desired amount of AOT/isooctane solution. Reverse micelles containing native lipase were prepared using the same procedure as pretreated lipase. 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.

2.5. Determination of lipase activity

Lipase activity was defined as the initial reaction rate, V (mol dm^{-3} s^{-1}), using olive oil as a water insoluble substrate. Reaction was initiated by adding an appropriate amount of olive oil into a pre-incubated micellar solution containing lipase. The reaction mixture was agitated by a magnetic stirrer at 500 rpm in an incubator at a constant temperature of 25 °C for exactly 20 min, as it was found that within this time range, free fatty acid production was linearly dependent on time [28]. The resultant fatty acid was analyzed by the Lowry technique [29]. The details of activity determination have been described elsewhere [28]. All data were given as the average of five replicate experiments and were reproducible within ±5%. In this study, all of the concentration terms were based on the total volume of the reverse micellar system unless otherwise specified.

2.6. Tryptophan fluorescence spectroscopy

Fluorescence emission spectra for both native and acetone treated *C. viscosum* lipases were measured in AOT reverse micellar systems at $w_0 = 10$. Total lipase concentration in reverse micellar systems was kept constant at 10 mg dm^{-3}. The emission spectra were recorded from 300 to 400 nm by a Hitachi F-3010 fluorescence spectrophotometer at an excitation wavelength of 280 nm, the selective excitation wavelength for tryptophan residues [30,31]. Emission and excitation slit widths were 5 nm. Spectra were uncorrected for instrument sensitivity, but the emission of blank reverse micelles (without lipase) was subtracted. Three-dimensional crystal structure [32] and amino acid sequence indicate that *C. viscosum* lipase contains 3 tryptophan, 10 tyrosine and 8 phenylalanine residues as fluorophores whose excitation wavelengths are 280, 257 and 270 nm, respectively. However, only tryptophan residues are sensitive in water or AOT/water/isooctane media.

2.7. Lipase stability

The reverse micellar solutions containing native or pretreated lipase was incubated at 25 °C, pH 8 and $w_0 = 10$ in the absence of substrate. After incubation, the samples were withdrawn at regular time intervals in order to measure the remaining enzyme activity by the addition of substrate (olive oil). The stability of lipase was expressed as the residual activity, which was calculated as a percentage of the original activity (considered 100%), obtained at $t = 0$ min incubation. The half-life of lipase was calculated directly from residual activity profiles.

3. Results and discussion

3.1. Pretreatment parameters

3.1.1. Effect of acetone content on enzyme activity

Fig. 1 shows that with the increase in acetone content, the lipase activity increases to a maximum value at an acetone volume fraction of 50%, and then decreases slightly. In general, when the enzyme is dissolved in water, the majority of its hydrophilic amino acids face toward the surface and hydrophobic amino acids toward the core. However, when the enzyme comes into contact with organic solvents, its hydrophobic amino acids are induced to the enzyme surface. It has been reported that 2-propanol–water favors a more hydrophobic-open conformation while pure water favors a less hydrophobic-closed conformation [33]. Thus, the activity enhanced by the pretreatment with acetone is attributed to the change in lipase conformation from a less hydrophobic-

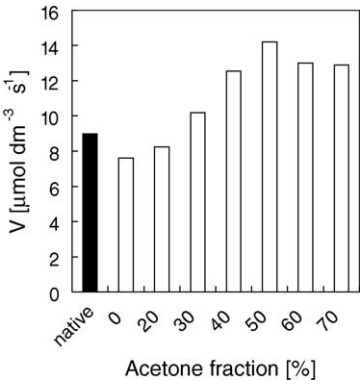

Fig. 1. The effect of acetone content in water–acetone solution on the activity of pretreated lipase. Pretreatment conditions: C_{lipase} = 1 mg/ml (in water–acetone), temperature during agitation = 4 °C, lipase agitation time 1 h, water–acetone solution pH 8, freeze-drying time 24 h. Activity assay conditions: C_{lipase} = 2 mg dm^{-3}, $C_{olive\ oil}$ = 0.055 mol dm^{-3}, C_{AOT} = 0.05 mol dm^{-3}, $w_0 = 10$, buffer pH = 8, C_{NaCl} (in buffer) = 0.3 mol dm^{-3}, reaction temperature = 25 °C.

closed form to a more hydrophobic-open one, which improves the interaction between lipase active site and substrate. Since the acetone favors a more hydrophobic-open conformation, the activity of lipase increases with the increase in acetone fraction until 50%. The later decrease in activity is due to the enzyme denaturation caused by the strong interaction with acetone, which leads to the precipitation of enzyme. In fact, the addition of more organic solvent to the media leads to considerable changes in the structure of enzymes such as the increase in β-sheet content, mainly at the expense of α-helix and β-turn [34,35]. The increased β-sheet content can be attributed to the aggregation of the enzyme. The results in Fig. 1 show this fact clearly.

It is evident from Fig. 1 that the activity of lipase, treated with only water (acetone 0%), is lower than that of native lipase. Enzymatic activity may be diminished by the process of dehydration during freeze-drying [36–38]. Acetone enhances the rigidity of the enzyme structure through the increase in the hydrophobic interactions between peptide chains; therefore, enzyme denaturation can be minimized during freeze-drying. Furthermore, acetone may act as a stabilizing additive that appears to inhibit the unfolding through freeze-drying.

3.1.2. Effect of pH on enzyme activity

The effect of water–acetone solution pH on the activity of treated lipase entrapped in AOT reverse micelles is shown in Fig. 2. The highest activity is obtained at pH 7.2, which is almost equal to the isoelectric pH (p*I*) of the lipase (7.3). At this pH, lipase has no net charge on its surface, and the interaction with other charged surfaces is minimal. The catalytic activity of enzymes is significantly dependent on the pH of the solution from which it is lyophilized [39]. Activity of lyophilized enzymes can be greatly enhanced, with the pH of aqueous solutions coincident with the optimum pH for the lipase activity in water [40,41]. As noted above, at isoelectric pH (i.e., 7.2) lipase has no net charge on its surface, and this non-ionization state is then retained during lyophilization as well as solubilization in AOT reverse micelles. Therefore, the interaction of pretreated lipase with the head groups of

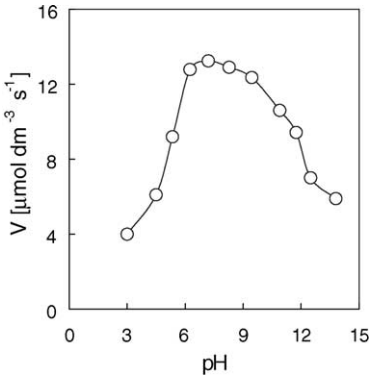

Fig. 2. The effect of water–acetone solution pH on the activity of pretreated lipase. Pretreatment conditions: lipase agitation time 1 h, acetone fraction in water–acetone solution 50%, freeze-drying time 24 h. Activity assay conditions are the same as those in Fig. 1.

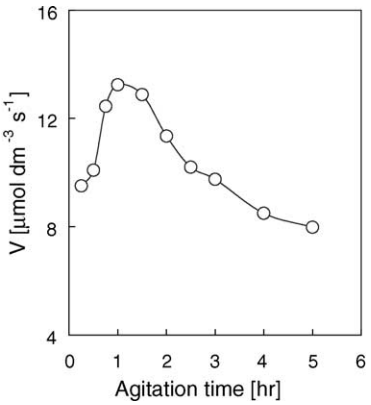

Fig. 3. The effect of lipase agitation time in water–acetone solution on the activity of pretreated lipase. Pretreatment conditions: water–acetone solution pH 7.2, acetone fraction in water–acetone solution 50%, freeze-drying time 24 h. Activity assay conditions are the same as those in Fig. 1.

AOT molecules is minimized [18] and lipase activity is maximized at this pH. At highly acidic or basic pH environments, the enzyme suffers from denaturation.

3.1.3. Effect of agitation time on enzyme activity

Fig. 3 indicates that with an increase in agitation time, lipase activity increases to a maximum value and then begins to decrease. The optimum agitation time is found to be 1 h. The lipase agitation time in water–acetone solution favors the exposure of hydrophobic amino acids to the surface. Thus, the concentration of hydrophobic amino acids on the lipase surface increases with the increase in agitation time, so it can be hypothesized that the increase in hydrophobic amino acids on the lipase surface enhances the affinity between lipase and the hydrophobic substrate. Affinity as the major driving force for the enzymatic catalysis depends on the binding energy between the enzyme and substrate [42]. In order to obtain proper binding, a greater net binding energy (high affinity between substrate and enzyme) is required. Therefore, it is reasonable to assume that the optimum agitation time corresponds to a state in which the affinity of treated lipase reaches a maximum. As a consequence, the active site of treated lipase can easily access the micellar interface as well as the substrate when it is solubilized in reverse micelles. At higher agitation times, the decrease in lipase activity may be attributed to lipase denaturation due to long-term interaction with acetone.

3.1.4. Effect of freeze-drying time on enzyme activity

The activation of lipase is highly sensitive to freeze-drying time, as it could cause a change in enzyme conformation induced by the process of dehydration. Water content of the final pretreated sample (lyophilized powder) has a significant effect on the activity of lipase [42–44]. In fact, protein requires water to maintain secondary and tertiary structures. If this water is removed, materials may be denatured with concomitant loss of some or all activity. Ru et al. [43] demonstrated that a direct correlation between water content and enzyme activity was obtained through the freeze-drying time.

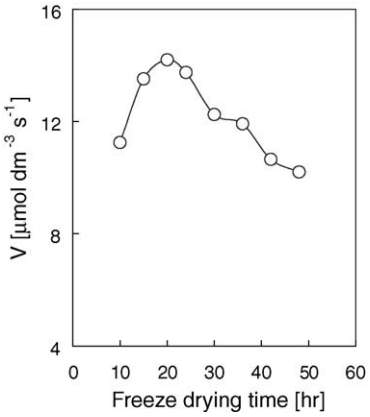

Fig. 4. The effect of freeze-drying time on the activity of *C. viscosum* lipase during pretreatment. Pretreatment conditions: lipase agitation time 1 h, acetone fraction in water–acetone solution 50%, water–acetone solution pH 7.2. Activity assay conditions are the same as those in Fig. 1.

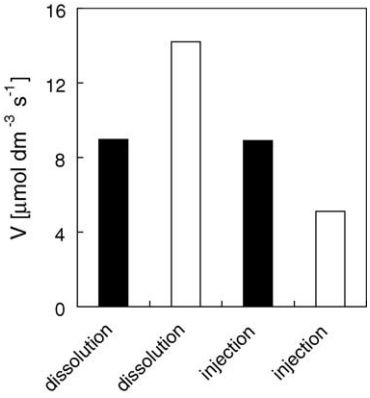

Fig. 5. The effect of lipase microencapsulation methods in AOT/water/isooctane reverse micelles. Symbols (■) and (□) represent the activity of native and acetone treated lipases, respectively. Pretreatment conditions: lipase agitation time 1 h, acetone fraction in water–acetone solution 50%, water–acetone solution pH 7.2, freeze-drying time 20 h. Activity assay conditions are the same as those in Fig. 1.

Fig. 4 shows that lipase activity increases with the increase in drying time until 20 h and then begins to decrease. This result is in good agreement with results reported in the literature [43], where it has been reported that the concentration of active site increases during the initial freeze-drying time, when sublimation of volatile components is still occurring. However, as freeze-drying time increases and water content decreases, the concentration of active site in freeze-dried samples decreases due to the removal of critical water from the active site [43]. It should be noted that dehydration-induced changes in secondary structures of proteins, accompanied by the increasing and decreasing in α-helix content and β-sheet content respectively, affect the catalytic activity in non-aqueous environments [37]. Moreover, at longer drying times, the water required for maintaining a catalytically active structure may be lost, resulting in less activation. Hence, in this study, the treated lipase attains an optimum structure to fully exert catalytic activity at a freeze-drying time of 20 h.

3.2. Lipase microencapsulation and its activity

To understand the principle of lipase conformational change, both the native and treated lipases are microencapsulated in AOT/isooctane reverse micelles by two different methods called the injection and dissolution methods. The activities obtained from each method are shown in Fig. 5. It is found that the method of lipase encapsulation has no effect on the activity of native lipase. However, when the treated lipase is encapsulated in AOT reverse micelles by the injection method, its activity decreases to a lower value than that of native lipase. A plausible explanation for this behavior is that when freeze-dried lipase is dissolved in water, it may return to its pre-acetone treatment conformation and thus its surface hydrophobic amino acids are further hidden in the lipase core. This hypothesis is also supported by the results obtained by Griebenov and Klibanov [36], where they have reported a lyophilization-induced reversible change in the structure of proteins. On the other hand, when lyophilized lipase powders are suspended directly in reverse micelles, the change in lipase conformation accompanied by the pretreatment is retained due to insufficient water and the hydrophobic state of micellar bound water. This observation further indicates that pretreatment leads to a change in lipase conformation by altering surface residues, which favor the exposure of binding sites (hydrophobic residues) in the open conformation.

3.3. Fluorescence spectroscopy of acetone treated lipase

Proteins contain several chromophores that absorb light in ultra-violet and infrared regions. Many chromophores called fluorophores can also display fluorescence. The conformational change in *C. viscosum* lipase by pretreatment with acetone was investigated, measuring fluorescence intensity.

The most important fluorophores of *C. viscosum* lipase are the aromatic rings of phenylalanine, tyrosine and tryptophan. Fluorophores display shifted spectra with increasing or decreasing protein surface charge, changes in wavelength of maximum absorbance (λ_{max}), and molar extinction coefficient. In general, lipase fluorescence emission is dominated by the contribution of tryptophyl. Conventional amino acid analysis frequently underestimates the content of this important amino acid, which is frequently involved in enzymatic catalysis. Fluorescence spectra of *C. viscosum* lipase also show that only tryptophan residue exhibits a distinctive peak at an excitation wavelength of 280 nm [30]; thus, our experiment was performed at an excitation wavelength of 280. There are three types of tryptophan residues (30, 209, 283) in lipase. Among them residues 209 and 283 are very close to the active site in any active conformation of *C. viscosum* lipase.

The fluorescence emission spectra for native and treated lipases in AOT/water/isooctane reverse micellar system are

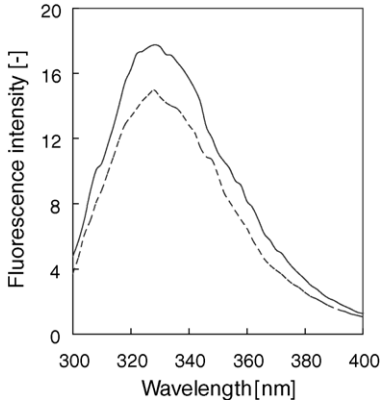

Fig. 6. Fluorescence spectra of native and treated lipases in AOT/water/isooctane reverse micelles. Symbols (- - -) and (—) represent the fluorescence intensity of native and acetone treated lipases respectively. Experimental conditions: *C. viscosum* lipase = 10 mg dm^{-3}, C_{AOT} = 0.05 mol dm^{-3}, C_{NaCl} = 0.03 mol dm^{-3}, pH = 8, w_0 = 10.

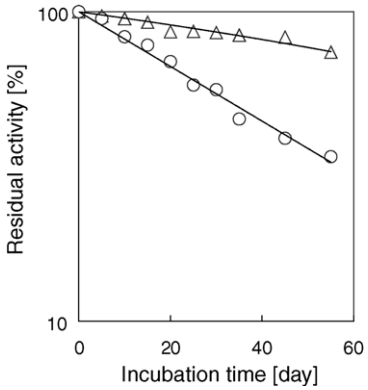

Fig. 7. Stability of lipase in semi-log plot. Symbols (◯) and (△) represent the stability of native and acetone treated lipase respectively. Storage conditions: *C. viscosum* lipase = 2 mg dm^{-3}, incubation temperature = 25 °C, w_0 = 10, C_{AOT} = 0.05 mol dm^{-3}, C_{NaCl} = 0.03 mol dm^{-3}, pH = 8. Activity assay conditions: $C_{olive\ oil}$ = 0.055 mol dm^{-3}, reaction time = 20 min and reaction temperature = 25 °C.

shown in Fig. 6. It is found that the fluorescence intensity of acetone treated lipase is higher than that of the native lipase. However, the maximum emission wavelength (λ_{max}) is the same (328 nm) for both lipases. It is well known that chromophores display shifted spectra of λ_{max} only upon increasing or decreasing polarity of their environment. In this study, the polarity of reverse micelles is unchanged for both native and treated lipases. Therefore, only a conformational rearrangement could cause the enhancement in fluorescence intensity. This increase in fluorescence intensity suggests that the pretreated lipase may have a better orientation, leading to more tryptophan (hydrophobic) residues on the surface. Since the active site of *C. viscosum* lipase is located in the hydrophobic amino acid region, it can easily access the micellar interface. As a result, the combination of active site with the substrate (olive oil) becomes more effective. Isabel del-Val and Otero [45] have reported that the change in tryptophyl fluorescence spectra is sufficient evidence of protein conformational change.

3.4. Stability of lipase

A major concern for the application of lipase to triglyceride hydrolysis is the stability in AOT/water/isooctane reverse micellar systems. In bioprocesses, it is a very important factor to have high activities as well as to maintain these activities during the elapsed time. Although the enzyme exhibits an appreciable activity in AOT reverse micellar systems, it is deactivated in reverse micelles [8,46–49]. A fast deactivation of *C. viscosum* lipase in AOT reverse micelles occurs due to the reversible denaturation process. Denaturation is probably due to two factors: first, the interaction between the head groups of AOT molecules and the enzyme results in deactivation [50], and secondly, partially hydrophobic bound water is an unfavorable environment for the lipase inside the water pool, which also inhibits the exposure of the active site of the lipase to the micellar interface [12].

In this study, the stability of *C. viscosum* lipase entrapped in AOT reverse micelles is increased significantly by pretreatment with acetone. From Fig. 7, it is evident that the pretreated lipase is very stable, retaining over 83% of its initial activity after 40 days. In contrast, native lipase retains only 43% of its initial activity after the same duration. It is also found from the residual activity profiles that the half-life of acetone treated lipase in AOT reverse micelles is 120 days, whereas the half-life of native lipase is 38 days. It may be assumed that the increased hydrophobic residues on the protein surface may improve its stability in reverse micellar systems, thus making the lipase more adaptable in partially hydrophobic micellar bound water, and also increasing the affinity between the lipase and the hydrophobic substrate. Vinogradov et al. [51] have reported that hydrophobic amino acids on the enzyme surface stabilize the enzyme against denaturation in organic media. In addition, the observed increase in lipase stability is attributed to the suppression of the interaction between lipase and AOT molecules because of the change in lipase surface residues.

4. Conclusions

The activity and stability of acetone treated *C. viscosum* lipase-catalyzed hydrolysis of olive oil was carried out in AOT/water/isooctane reverse micelles. A surprising dependence of catalytic activity of the treated lipase on the pretreatment conditions was observed. Lipase microencapsulation methods in AOT reverse micelles supported the hypothesis of a change in lipase conformation. Fluorescence analysis suggested that the treated lipase may have a better orientation, which leads to exposure of more hydrophobic amino acids to the lipase surface. Since the active site of *C. viscosum* lipase is located in the hydrophobic amino acid region, it can easily access the micellar interface, and comes into contact with hydrophobic substrate more effectively. Acetone treated lipase

exhibited higher stability in AOT reverse micelles than that of native lipase. The half-life of acetone treated lipase was 3.2 times higher than native lipase.

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