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# Structural and catalytic aspects of cutinase in w/o microemulsions

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**Abstract** Structural and catalytic properties of cutinase were studied in bis(2-ethylhexyl) sodium sulfosuccinate (AOT)-isooctane microemulsion systems. The effect of the water content of the microemulsions on the cutinase activity on an esterification reaction of lauric acid with pentanol showed that cutinase followed a bell-shaped profile presenting a maximum at $w_o = 9$, with $w_o = [H_2O]/[AOT]$. Kinetic studies allowed the determination of the apparent parameters $K_m$ and $V_{max}$. Electron paramagnetic resonance (EPR) spectroscopy studies of active site labeled cutinase in microemulsions with varying $w_o$ values showed that in all microemulsions, the mobility of the label is higher than in the aqueous solution. Furthermore, it was found that the maximum of the enzyme activity did not correspond to a reduced active site mobility. Up to $w_o = 9$ there was an increase of both activity and active site mobility. As the water content of the system became higher, the mobility of the bound spin label further increased whereas the enzymatic activity dropped considerably.

**Key words** Electron Paramagnetic Resonance, EPR – microemulsions – reverse micelles – AOT – lipase – esterification

## Introduction

Microemulsions are fine dispersions of water in an organic solvent stabilized by surfactant molecules [1]. The main property of these systems is the coexistence of two completely different microphases in an apparently macrohomogeneous solution. The use of microemulsions to solubilize enzymes in organic solvents has attracted considerable interest in the past decade [2]. A large variety of enzymes have been successfully encapsulated in reverse micellar systems retaining their catalytic activity [3]. The interior of the microemulsions acts like a microreactor that provides a favourable aqueous microenvironment for enzyme activity, as well as an enormous interfacial area through which the conversion of hydrophobic substrates can be catalyzed.

Enzyme containing microemulsion systems are optically clear. Thus, spectroscopic techniques, such as ultraviolet absorption (UV), fluorescence, circular dichroism (CD), electron paramagnetic resonance (EPR), nuclear magnetic resonance (NMR), can be applied for monitoring the conformational changes that may occur in enzyme molecules upon their solubilization in microemulsions [4].

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Moreover, in these systems it is possible to precisely control the water microenvironment (interfacial/bulk) and therefore the size and structure of the system, ranging from enzyme- containing large reverse micelles, to a situation where the enzyme is restricted in very poor water containing reverse micelles. The size of the micelles is regulated by varying the water/surfactant molar ratio, $w_o$ [5].

Despite the number of studies in microemulsions concerning structure/function relationships of enzymes, using spectroscopic techniques, lipases have not so far attracted considerable attention. Lipase is an enzyme with great technological interest, inexpensive and widely used in the development of industrial applications in various sectors, such as detergents, oils and fats, dairy, cosmetics and pharmaceuticals [6]. In addition to the hydrolysis of triglycerides to glycerol and free fatty acids, lipases can be used for the catalysis of esterification and transesterification reactions in low water content media [7–9]. This catalytic process is heterogeneous and can be favored by the use of microemulsions. Lipases are active almost exclusively near interfaces in a classical heterogeneous procedure [10].

In this work cutinase from *Fusarium solani*, encapsulated in water in oil microemulsions has been used as a model system, where its catalytic behavior was related to the enzyme conformation as followed by EPR spectroscopy. The structure of cutinase has been solved showing that it is a compact 20 kDa enzyme containing a small flap and an accessible active site serine residue. Cutinase which catalyzes the hydrolysis of cutin, the insoluble polymer covering the surface of plants, can also efficiently catalyze the hydrolysis of triglycerides, and is considered as a lipase [11, 12]. It is also known that cutinase is able to interact with liquid–water interfaces, but does not exhibit interfacial activation. A consequence of the interaction of the enzyme with the lipid phase is the modification of the water distribution and behavior at the interface and the active site microenvironment.

Electron paramagnetic resonance spectroscopy can be used as a technique for the understanding of the nature of the catalytic activity of an enzyme. Probing the active site of an enzyme molecule with a covalently bound specific spin label, important information can be derived on the microenvironmental level concerning changes in conformation, microviscosity, and micropolarity [13, 14].

The present study of the catalytic behavior of cutinase in relation to its active site conformation in restricted and controlled hydration levels, can elucidate the role of water on this enzymic process. For this, the microemulsion system formulated with the anionic surfactant AOT in isooctane [5], was employed to probe the catalysis of the esterification reaction of a fatty acid with alcohols [15, 16].

## Experimental

### Materials

*Fusarium solani pisi* recombinant cutinase was provided by Dr. Egmond, Unilever Research Laboratorium, Vlaardingen. The enzyme preparation had a hydrolytic activity on *p*-nitrophenyl butyrate of 356 u/mg of solid at pH 7.4, 25 °C. One unit is defined as the amount of cutinase that produces 1 mmol *p*-nitrophenol per minute at the above experimental conditions. The spin label 4-(ethoxy-fluorophosphinyloxy)-2,2,6,6-tetramethylpiperidine-*N*-oxyl (EFP-TEMPO) was from Sigma. Surfactant bis(2-ethylhexyl) sodium sulfosuccinate (AOT), *p*-nitrophenyl butyrate and lauric acid were purchased from Sigma. Isooctane was from Merck and pentanol was from Ferak, Berlin. All chemicals were of the highest available degree of purity and twice-distilled water was used throughout this study.

### Methods

#### *Spin labeling of cutinase*

The active site serine residue of cutinase was spin labeled by the phosphonylating spin label EFP-TEMPO according to a modified procedure of Morrisett and Broomfield [17]. 10 mg of cutinase were dissolved in 1.3 ml of 0.1 M sodium acetate buffer pH 5.5. A 0.39 M stock solution of the spin label in acetonitrile was prepared. The spin-labeling reaction was started by adding to the cutinase solution appropriate amounts of EFP-TEMPO so as to obtain a final tenfold molar excess of spin label to enzyme. The reaction was followed by monitoring the cutinase activity as regards the hydrolysis of *p*-nitrophenyl butyrate. Within 3.5 h only 5% of the initial enzyme activity remained. The unreacted spin label was then removed by extensive dialysis against a 55 mM glycine/HCl, pH 3.5 solution. The spin-labeled enzyme solution was removed from the dialysis bag and stored in a freezer. The concentration of the spin-labeled enzyme in the final solution was determined by measuring the absorbance at 280 nm ($E_{280\ \mathrm{nm},\ 0.1\%,\ 1\ \mathrm{cm}} = 0.713$).

#### *Preparation of microemulsions*

Cutinase containing microemulsions were prepared by adding appropriate amounts of an aqueous enzyme solution to a solution of 0.1 M AOT in isooctane. Enzyme solubilization was achieved by gentle shaking within less than 1 min. The total amount of water was adjusted to give

the desired value of the molar ratio $w_o = [H_2O]/[AOT]$. The water content of the initial stock solution AOT/isooctane was periodically checked by Karl Fischer titrations. The amount of water (in general less than 0.5% w/w) was taken into consideration in the calculation of the total water content.

*Activity measurements*

The activity of cutinase in 0.1 M AOT reverse micelles was determined by monitoring the esterification of lauric acid with pentanol at a temperature of 25 °C. The esterification reaction was started by addition of pentanol in a cutinase containing microemulsion where lauric acid had already been solubilized. The total volume of the reaction was 1 ml and all the concentrations of the reactants are referred to this volume. The pH of the dispersed aqueous phase is supposed to be that of the initial buffer solution, 0.1 M Tris/HCl pH 9. The initial reaction rates were determined spectrophotometrically, by measuring the depletion of fatty acid as described elsewhere [7]. Curves of product formation as a function of time were linear over a period of about 10 min, thus allowing a reliable determination of the initial slopes. All measurements for the determination of the kinetic parameters were carried out in duplicate.

Stability measurements

*Stability of cutinase*

Cutinase containing AOT reverse micelles of three different $w_o$ values ($w_o = 5$, 9 and 20) were prepared and incubated at 25 °C. The overall enzyme concentration was kept constant 0.16 mg/ml in all cases. The total volume of the reaction medium was 5 ml. The cutinase stability was evaluated by determining the initial reaction rate for the esterification of lauric acid (97 mM) with pentanol (300 mM) in AOT microemulsions, as follows: At given time intervals 0.6 ml of the cutinase containing microemulsion were withdrawn and added to a microemulsion of the same $w_o$, containing both of the substrates. The total volume of the reaction was 0.8 ml. The depletion of lauric acid was minitored as in the case of the enzyme activity measurements.

*Stability of spin-labeled cutinase*

Reverse micelles of different $w_o$ values ($w_o = 5$, 9, 15 and 20) containing spin-labeled cutinase were prepared and incubated at 25 °C. The final concentration of spin-labeled enzyme in the dispersed aqueous phase was kept constant for all the $w_o$ values tested. At given time intervals samples of 0.7 ml were taken out and their EPR spectra were recorded. For every $w_o$ value, three independent sets of experiments were carried out at constant time intervals.

*Electron Paramagnetic Resonance (EPR) measurements*

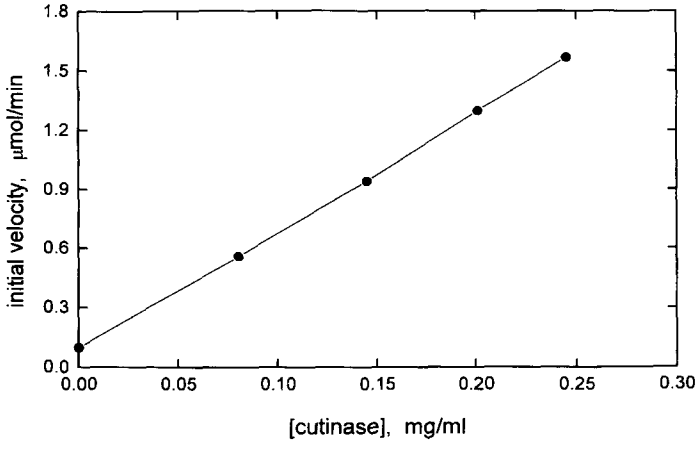
EPR spectra were recorded at room temperature using a Bruker ER 200 D spectrometer operating at X-band. Spectra were accumulated and treated using the DAT-200 software for PC (University of Lubeck). The samples were contained in an E-248 cell. Typical settings were: field, 3471 G; gain, 20 000; time constant, 1 s; microwave power, 7.5 mW; microwave frequency, 9.76 Ghz; modulation amplitude, 2.5 G.

## Results and discussion

Activity studies

The effect of enzyme concentration on the reaction rate, when the esterification of lauric acid with pentanol was considered, is shown in Fig. 1. A linear dependence is observed which is consistent with a kinetically controlled enzymatic reaction. The effect of the water content of the microemulsions on the cutinase activity, was studied for the above-mentioned esterification. The reaction was carried out at 25 °C and pH 9, values corresponding to the optimum ones as determined by Sebastiao et al. [18]. Figure 2 shows the variation of the specific activity of

**Fig. 1** Initial velocity of esterification of lauric acid by pentanol as a function of cutinase concentration. [Lauric acid] = 100 mM, [Pentanol] = 290 mM, pH 9, $T = 25$ °C



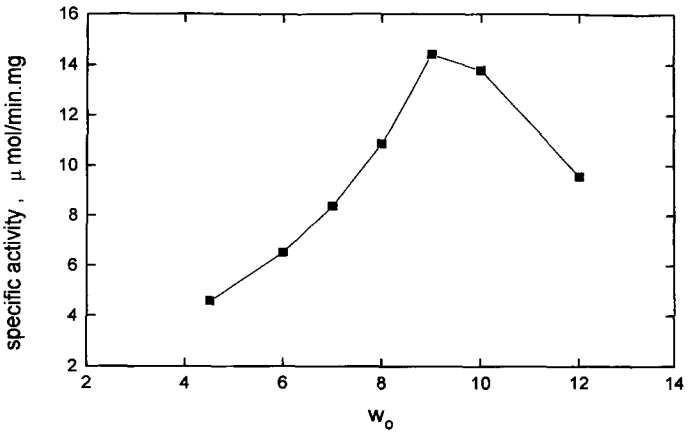



**Fig. 2** Esterification of lauric acid by 1-pentanol, catalyzed by cutinase, in AOT reverse micelles. Variation of the specific activity of the esterification reaction as a function of the water content as expressed by $w_o$ ($w_o = [H_2O]/[AOT]$). [Lauric acid] = 100 mM, [Pentanol] = 290 mM, pH 9, $T = 25\,^{\circ}C$

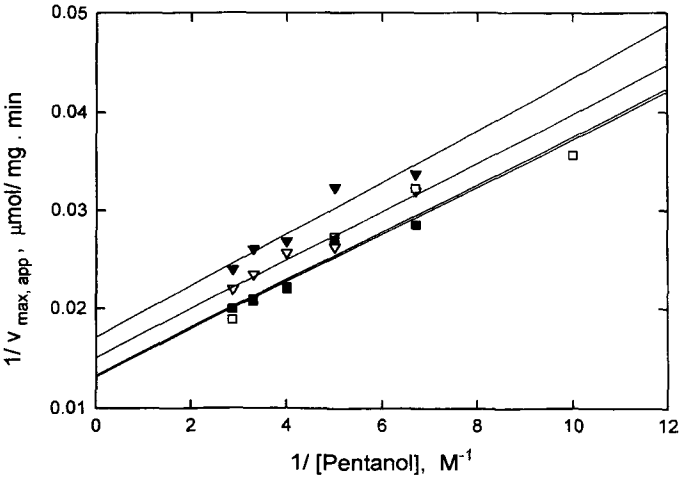
esterification as a function of the water content, in terms of $w_o$ values. It can be seen that cutinase activity follows a bell-shaped profile presenting a maximum at $w_o = 9$ for the given microemulsion system. The appearance of an optimum seems to be due to a number of factors. Several models have been proposed to explain the dependence of enzyme activity on $w_o$. According to a model proposed by Bru et al. [19] the bell-shaped pattern is characteristic of enzymes that express maximal catalytic activity in the region of the bound water. Structural studies on the AOT-based microemulsions have shown that the dispersed aqueous phase constitutes spherical droplets, surrounded by a monolayer of surfactant molecules [5]. By varying the $w_o$ value, the radii of the reverse micelles are varied by several Å [20]. Each AOT molecule needs 2–4 water molecules for the hydration of its head group and 6 water molecules for each counter ion, $Na^+$ [4]. After hydration of the surfactant head groups, excess water is used for enzyme hydration which seems to be complete at $w_o$ around 9. Water molecules that hydrate the polar heads are subject to different forces from those of the bulk water. Hydrogen bonding and electrostatic attractions with the charged polar heads originate more structured water than the bulk one. These changes in the vicinity of the enzyme possibly affect its conformation and thus its catalytic activity and stability. The effect of the water content on the dynamics of the active site of cutinase as indicated by EPR spectroscopy will be discussed in detail later.

Apart of the conformational changes of the protein upon solubilization in reverse micelles, bell-shaped dependence of the activity can be also attributed to the diffusion of the substrates in the microheterogeneous system. A kinetic model based on the different diffusion of the substrates between and within enzyme containing reverse micelles has been presented by Maestro [21] in order to explain the appearance of an optimum $w_o$ in the activity of many enzymes.

On the other hand, it has been pointed out in the literature that in many cases bell-shaped curves are explained by the use of nonsaturating substrate concentrations [22]. In our case, where an esterification reaction was studied and two substrates were involved, calculation of $K_{cat}$ parameters for every $w_o$ value tested was practically not possible. Nevertheless a bell-shaped dependence was observed, both when saturating and nonsaturating substrate concentrations were considered. According to the above observation, the appearance of a maximum at $w_o = 9$ is rather due to the hydration degree of the protein and not diffusional and/or partitioning phenomena.

To elucidate the mechanism of the esterification reaction catalyzed by cutinase in AOT reverse micelles, a kinetic study was undertaken using pentanol and lauric acid as substrates. Figure 3 shows the variation of reciprocal initial velocity as a function of pentanol reciprocal concentration of different fixed lauric acid concentrations (85, 120, 150, 200 mM) at $w_o = 9$, pH 9. The appearance of parallel lines, as shown in Fig. 3, suggests that the process is characterized by a ping–pong bi–bi mechanism, a mechanism in which a product is released between the addition of two substrates [23]. The same mechanism has been proposed for similar reactions catalyzed by various lipases in microemulsions and other nonconventional media [15].

**Fig. 3** Double reciprocal plot of the initial velocity of esterification of pentanol by lauric acid as a function of alcohol concentration, at different fixed concentrations of acid: 85 mM (▼), 120 mM (▽), 150 mM (□), 200 mM (■), in 0.1 M AOT/isooctane microemulsions, $w_o = 9$, pH 9, $T = 25\,^{\circ}C$



According to the mechanism the enzyme reacts with lauric acid to form a lipase–acid complex. This complex is then transformed to a carboxylic–lipase intermediate and water is released. This is followed by an attack of pentanol on this intermediate to form the final pentyl laurate. The kinetic constants of the esterification reaction were determined as follows: by plotting the reciprocal values of the maximum apparent velocity, $1/V_{max,app}$, issued from Fig. 3, vs. the reciprocal concentrations of lauric acid a straight line is obtained. From the slope and the intercept a $K_m$ (lauric acid) value of $60 \pm 10$ mM and a $V_{max}$ value of $100.1 \pm 9.6$ $\mu$mol min$^{-1}$ mg$^{-1}$ can be calculated. By plotting the reciprocal value of apparent pentanol Michaelis constant, $1/K_{m,app}$ (pentanol), as derived from Fig. 3, vs. the reciprocal concentrations of pentanol, a $K_m$ (pentanol), value of $208 \pm 10$ mM is obtained. The above kinetic constants are apparent ones since the environment of the reaction medium is not homogeneous.

The value of the Michaelis constant, $K_m$ (lauric acid), determined in the above esterification is quite similar to that found for oleic acid when the esterification of oleic acid with hexanol catalyzed by cutinase in the same microemulsion system, was considered [18]. Comparable $K_m$ (acid) values have been reported for the esterification of lauric acid with menthol catalyzed by *Penicillium simplicissimum* lipase [15] and the esterification of lauric acid with octanol catalyzed by lipase from *Pseudomonas cepacia* in AOT reverse micelles [8]. It seems that fatty acids with long aliphatic chains originate similar affinities with lipases when solubilized in microemulsions. These fatty acids, as it has been shown in previous SAXS studies [24] are mainly located on the surfactant monolayer and oriented to the continuous oil phase.

As far as the Michaelis constant of the pentanol is concerned, the relatively high value observed is quite expected if one takes into consideration the location of the alcohol in the system. Interfacial titrations have shown that pentanol participates in the interface and its solubility in the aqueous phase is almost negligible [25].

## Stability studies

The stability of cutinase in AOT reverse micelles of different $w_o$ values was investigated over a period of 24 h at 25 °C, pH 9. Figure 4 shows the variation of the % enzyme activity as a function of incubation time. A fast inactivation of cutinase after solubilization in reverse micelles can be observed. According to Fig. 4, the rate of inactivation strongly depends on the $w_o$ value of the microemulsion system. Namely, the stability of cutinase is decreased as the $w_o$ of the reverse micelles is increased. The most rapid loss of activity is observed for $w_o = 20$. Fast inactivation of cutinase at $w_o = 20$ has been also reported by Melo et al. [26] when a triglyceride hydrolysis reaction was monitored. A similar behavior has been observed previously for other enzymes in reverse micelles and was attributed to the denaturant effect of the anionic surfactant AOT [27, 28].

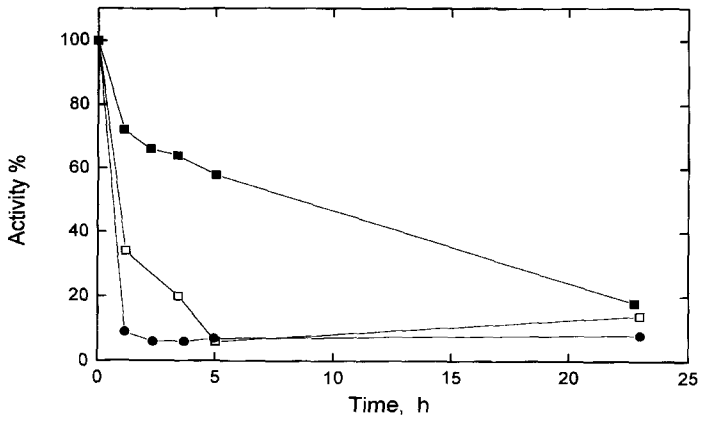



**Fig. 4** Effect of $w_o$ on cutinase stability in 0.1 M AOT/isooctane microemulsions, at pH 9, $T = 25$ °C. $w_o = 5$ (■), $w_o = 9$ (□), $w_o = 20$ (●).

## Electron paramagnetic resonance studies

In order to further elucidate the effect of the water content of the reaction medium $w_o$ on the cutinase activity and stability, we have undertaken an EPR spectroscopic study. We have used the organophosphorus spin label EFP-TEMPO which is highly specific for the nucleophilic serine residue at the active site of many enzymes [29]. Cutinase is a serine esterase containing the classical catalytic triad, Ser, His and Asp, found in the serine hydrolases [11]. Unlike other lipases the active site serine residue of cutinase is not buried under surface loops but is relatively accessible to the solvent. Since spin labels are sensitive to small changes occuring in the microenvironment around them the EPR spectra of spin-labeled cutinase are expected to report changes in the microenvironment of the active site.

The EPR spectra were recorded in the above-described microemulsions with varying $w_o$ values. Figure 5 shows the free spin label spectrum (a), as well as the spectra of the spin-labeled cutinase obtained in aqueous solution (b) and in AOT reverse micelles of two different $w_o$ values (c, d). The spectrum of the free spin label is characterized by three sharp peaks of approximately equal amplitude. This spectrum indicates a rapidly rotating spin label. When the label is covalently attached to the active site of the enzyme, the spectra are considerably changed. The peaks are broadened and in some cases the high field peak

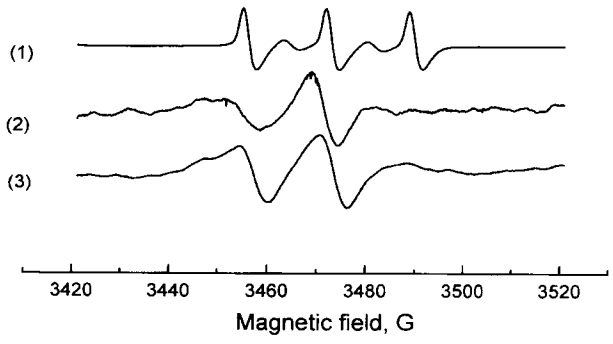



**Fig. 5** EPR spectra of free spin label (1) and of spin-labeled cutinase in aqueous solution (2) and in reverse micelles of $w_o = 9$ (3).

almost disappears. These changes are indicative of a hindered motion of the label and depend on the enzyme microenvironment.

An analysis of the EPR spectra of the nitroxide radical permits the calculation of its rotational correlation time when covalently bound to the active site of the enzyme. The line amplitudes of the EPR spectra are necessary for the calculation of this value. A short correlation time is indicative of a rapid motion and a longer correlation time is indicative of a restricted motion. When the rotational diffusion of the label is too slow, $t_R > 10^{-9}$ s, then rotation correlation times cannot be calculated with equations proposed for the rapid-motional region and a special treatment is needed [30]. In our case we have EPR spectra in both rapid and slow-motional regions. In order to obtain a more uniform idea of the dynamics of the active site we have used a rather empirical parameter [31]. This parameter is the rate of the intensities of the low to the center field lines, $h_{+1}/h_0$, of the spectrum and is indicative of the mobility of the label. Table 1 shows the values of this parameter for the free spin label and for the labeled cutinase in aqueous and reverse micellar solutions of different $w_o$ values. It is seen that in all microemulsions, except for those with very low water content, $w_o = 5$, the mobility as expressed by the ratio $h_{+1}/h_0$, is higher than in the aqueous solution. By comparing the EPR spectra of spin-labeled cutinase both in aqueous solution and reverse micelles of $w_o = 5$ it is obvious that there are no dramatic changes on the active site conformation. As $w_o$ value increases up to $w_o \sim 20$, the mobility of the label is constantly increased whereas for higher $w_o$ values it remains almost constant.

The above-mentioned results diverge from earlier work on spin-labeled enzymes in reverse micelles. When EPR studies of a serine protease such as *a*-chymotrypsin spin-labeled with EFP-TEMPO were performed in AOT reverse micelles, no major changes in the active site

**Table 1** Values of the ratio $h_{+1}/h_0$ for the free spin label and for spin-labeled cutinase, in aqueous solution and 0.1 M AOT/isooctane microemulsions

| System | $h_{+1}/h_0$ |
|---|---|
| Free spin label | 1 |
| Spin-labeled cutinase in aqueous solution | 0.50 |
| Spin-labeled cutinase in AOT microemulsions | |
| $w_o = 5$ | 0.50 |
| $w_o = 6$ | 0.69 |
| $w_o = 7$ | 0.71 |
| $w_o = 8$ | 0.64 |
| $w_o = 9$ | 0.79 |
| $w_o = 10$ | 0.75 |
| $w_o = 13$ | 0.95 |
| $w_o = 21$ | 1.04 |
| $w_o = 25$ | 1.11 |
| $w_o = 31$ | 1.13 |
| $w_o = 40$ | 1.14 |

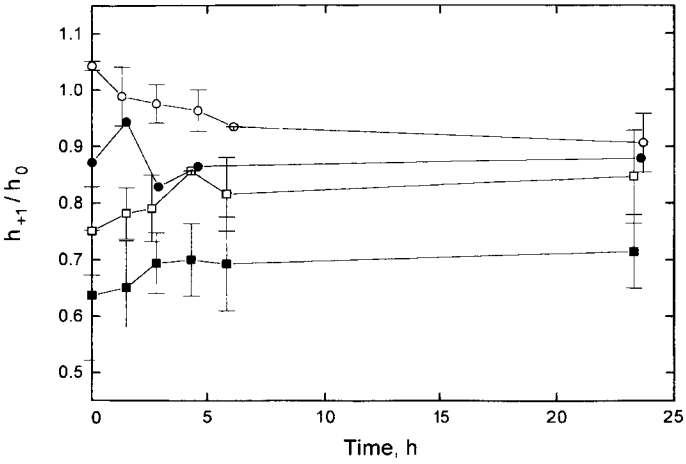



**Fig. 6** Effect of $w_o$ on the ratio $h_{+1}/h_0$ of spin-labeled cutinase as a function of incubation time. $w_o = 5$ (■), $w_o = 9$ (□), $w_o = 15$ (●), $w_o = 20$ (○).

mobility were observed on going from aqueous solution to reverse micelles of high $w_o$ values [14]. The same result was reported by Marzola et al. [32], who found that the rotational mobility of spin-labeled *a*-chymotrypsin changed little with $w_o$ when entrapped in AOT reverse micelles. Kommareddi et al. [33] reported changes in active site dynamics of spin-labeled *a*-chymotrypsin only at very low water content. However, Belonogova et al. [34] reported that spin-label mobility goes through a pronounced minimum at $w_o$ 15 to 20. It was observed that the dependence of the mobility of the spin label is related to the pattern of the variation of the catalytic activity of *a*-chymotrypsin with varying the degree of hydration of the micelles. When an extensive study of spin-labeled papain was performed in

AOT-based microemulsions, a higher structural rigidity of the active site was corresponding to a higher catalytic activity [35].

In the case of spin-labeled cutinase, the maximum of the enzyme activity does not correspond to a reduced active site mobility. Up to $w_o \sim 9$ there is an increase of both activity and active site mobility. As the water content of the system becomes higher, the mobility of the bound spin label further increases whereas the enzymatic activity drops considerably.

The increase of the $h_{+1}/h_0$ ratio with increasing water content is probably due to the destabilizing effect of the anionic surfactant AOT. As $w_o$ increases, the aggregation number of the micelles becomes higher. When a molecule of spin-labeled cutinase is entrapped in a reverse micelle of high aggregation number, its conformation becomes less rigid, probably due to the presence of more AOT molecules in the vicinity of the protein. As reported previously, only minor loss of activity is observed upon solubilization of cutinase in microemulsions based on the cationic surfactant cetyltrimethylammonium bromide (CTAB) [36]. On the contrary, the anionic AOT seems to have a strong inhibitory effect depending on the $w_o$ of the system.

In order to further investigate the denaturant effect of the anionic surfactant, a stability study of spin-labeled cutinase in reverse micelles of different $w_o$ values was undertaken. Figure 5 shows the variation of the ratio $h_{+1}/h_0$ as a function of incubation time. A strong effect of the preincubation time on the spin-label mobility, depending on the hydration degree of the micelles, is observed. For $w_o$ values of 5 and 9, there is an increase of the active site mobility increasing incubation time. For higher water content, $w_o = 15$, no considerable changes are observed. Finally, for $w_o = 20$, there is a remarkable decrease of the spin-label mobility. This differentiated behavior of the cutinase active site mobility for various $w_o$ is rather unexpected. Nevertheless, after a certain time of incubation, all the above mobilities, as expressed by the $h_{+1}/h_0$ ratio, seem to tend to the same value. It is interesting that the mobility of the spin label, regardless the $w_o$ of the incubation, finally tends to that same value of $h_{+1}/h_0$, which corresponds to $w_o$ around 9, where the optimum activity was encountered.

The activity and stability results presented above can be partly explained by the denaturant effect of the anionic surfactant. The structure at the active site is more flexible and fragile than other parts of the enzyme and thus is more sensitive to the effect of denaturant. A possible invasion of AOT into the active site or surfactant adsorption onto the enzyme surface may be the cause of protein unfolding. Conformational changes of the protein due to the denaturant effect of the surfactant seem to occur. The unfolding process is possibly very slow and irreversible, leading to a situation where the structure of the whole molecule is more relaxed and the active site fluctuates more freely. On the contrary, as regards the protein activity, when the water content of the system exceeds a certain limit and probably due to the presence of AOT, an almost complete inactivation occurs.

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