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Triglyceride Hydrolysis and Stability of a Recombinant Cutinase from *Fusarium solani* in AOT-*iso*-octane Reversed Micelles

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ABSTRACT

A recombinant cutinase from *Fusarium solani* was encapsulated in AOT reversed micelles. Physicochemical parameters of the system were optimized relative to triolein hydrolysis. Kinetic studies of triglyceride hydrolysis showed a decrease in specificity with increase of the acyl chain length. Stability of cutinase in the system under study is lower than in aqueous solution and decreases with increase in the water content in the system (W_0 = $[H_2O]/[AOT]$). The products of triolein hydrolysis had little effect on the cutinase stability. Although glycerol did not alter the stability, oleic acid decreases the enzyme stability. The increase in log *P* of solvent (from *iso*-octane to *n*-dodecane) decreased the stability. Deactivation profiles were fitted with the Henley and Sadana model (*1*).

Index Entries: Recombinant cutinase; reversed micelles; triglyceride hydrolysis; stability.

INTRODUCTION

Reversed micelles are a suitable medium to promote biocatalysis in organic media. The solubilization of the aqueous enzyme solution in the water pool of reversed micelles allows the retention of catalytic activity because of the shell formed by the surfactant molecules, which protects

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the enzyme against denaturation by the organic solvent. Such enzymatic systems create a microenvironment that provides a particular reaction medium, the protein molecule being in an optimal localization corresponding to its nature.

The utilization of this system in order to study the enzymatic hydrolysis of fats and oils provides an interfacial area of about 100 m^2/mL, which is much higher than that of other conventional biphasic biocatalytic systems (*2*). It also allows a conventional kinetic approach, since the substrate is in a monomeric form. From the point of view of application, one of the most important questions is the enzyme stability in this particular reaction medium.

Cutinases are a group of hydrolytic enzymes capable of degrading the insoluble lipid polyester matrix, i.e., cutin, which covers plant surfaces. They belong to the class of serine esterases, containing the classic catalytic triad serine, histidine, and a carboxyl group (*3*), and are able to hydrolyze a wide variety of synthetic esters and triglycerides (*3,4*). Cutinase, however, differs from classical lipases in that no flap exists in its structure (*5*) and no measurable interfacial activation around the critical micellar concentration (CMC) of the tributyrin substrate is observed (*4*).

In this article we report the behavior in AOT-*iso*-octane reversed micelles of a recombinant cutinase, from *Fusarium solani* overproduced in *Escherichia coli* (*4*). The optimization of the physicochemical parameters of the reversed micellar system to quantify the cutinase hydrolytic activity on triolein is carried out and the kinetic constants for the hydrolysis of triglycerides with varying acyl chain lengths are calculated. The effect of the physicochemical parameters of the system and the presence of the products of triolein hydrolysis are also studied in relation to the stability of the cutinase.

MATERIALS AND METHODS

Chemicals

Recombinant cutinase was a gift from CORVAS International N.V. (Gent, Belgium). This preparation was obtained as a lyophilized powder of over 95% purity (w/w).

Sodium-di-2-ethylhexyl sulfosuccinate (AOT) (99% purity) and L-α-phosphatidylcholine (64%) were obtained from Sigma (St. Louis, MO); toluene (99.7%) and *iso*-octane (99.5%) from Riedel-de-Haen; *n*-dodecane (>99%), oleic acid (extra pure), and glycerol (86–88%) from Merck (Darmstadt, Germany); triolein (~65%) from BDH; tricaprylin (97%), trilaurin (>97%), and trimyristin (97%) from Fluka (Buchs, Switzerland). All salts were of analytical reagent grade.

Preparation of Reversed Micelles and Activity Assays

Reversed micelles containing cutinase (0.1 mg/mL) were formed by adding appropriate amounts of an aqueous solution of cutinase to a solution of 0.1*M* AOT in *iso*-octane.

The activity assays, both in reverse micelles and in aqueous solution, were performed in a stirred batch reactor with a water jacket, and samples were removed at given time intervals. The variability of each activity value was less than 5%.

For the optimization of the physicochemical parameters (W_0, pH, molarity, and temperature) of the reverse micellar system, triolein, was added as substrate (0.1*M* final concentration) to the reactor immediately after the reversed micellar solution became clear. The optimization studies of W_0 ($[H_2O]/[AOT]$) and pH was carried out at 30°C and in 0.05*M* buffer solution (before the microencapsulation). The pH_{st} values (pH of the stock solution) are those of the aqueous buffer, from which the micellar solutions were prepared.

To calculate the kinetic constants for the hydrolysis of triglycerides, one solution of protein-free reversed micelles, with the appropriate W_0 and amount of substrate was added instead of the addition to triolein. This procedure enables controlled variations in substrate concentrations while maintaining the AOT and protein concentrations constant.

The activity assays in aqueous solution were done with 0.002 mg/mL of cutinase in a 0.05*M* of Tris-HCl or carbonate-bicarbonate buffer with 0.23% (wt/vol) of L-α-phosphatidylcholine to improve the emulsion properties. Triolein (0.1*M*) was used as the substrate.

The spectrophotometric method of Lowry and Tinsley (*6*) was used to quantify the formation of the fatty acid during the enzymatic reaction in aqueous solution and reverse micelles.

Stability Assays

Reverse Micelles

The reversed micellar solution (0.1*M* AOT and 50 m*M* buffer) containing the protein (0.2 mg/mL) was incubated at a given temperature and 5-mL samples were taken out at given time intervals and used to start a new reaction with triolein.

Aqueous Solution

The aqueous solution of cutinase (0.2 mg/mL of cutinase in 50 m*M* Tris-HCl buffer, pH 8.6) was incubated at 30°C. Samples of 50 μL were taken out at given time intervals and mixed with 5 mL of 50 m*M* Tris-HCl buffer, pH 8.6 with 0.23% (wt/vol) of L-α-phosphatidylcholine. Triolein (0.1*M*) was added to start a new reaction.

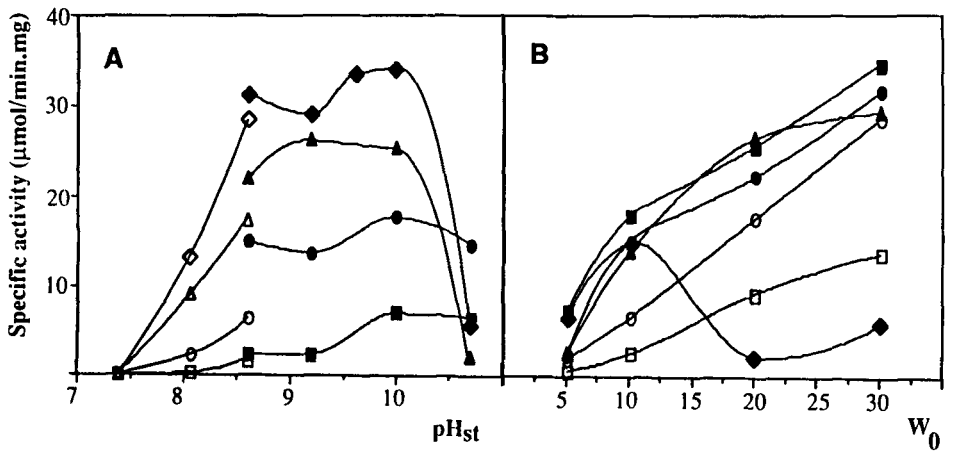

Fig. 1. Effect of pH and W_0 on triolein hydrolysis by cutinase in reverse micelles of AOT in *iso*-octane. (**A**) pH profile at four W_0 values: □ ■ W_0 = 5; ○ ● W_0 = 10; △ ▲ W_0 = 20; ◇ ◆ W_0 = 30. (**B**) Dependence of specific activity on the content of water solubilized in the system at five pH values: □ pH 8.07; ○ pH 8.6; ● pH 8.6; ▲ pH 9.2; ■ pH 10; ◆ pH 10.7. The empty symbols referred to values obtained with 0.05*M* Tris-HCl buffer and the full symbols with 0.05*M* carbonate-bicarbonate buffer.

To the application of the Henley and Sadana deactivation model (*1*), a nonlinear regression procedure with the Marquadt method of interactive convergence, was used.

RESULTS AND DISCUSSION

Optimization of the Physicochemical Parameters

Optimization of W_0 and pH

The pH profile was defined at four W_0 values (Fig. 1A) and shows a maximum activity around 9.5–10. This optimum is shifted about one unit to higher pH values relative to aqueous solution, where the optimum occurs at around 8.6 (data not shown). This is probably because of lower intramicellar pH values (*7*). The hydrolytic activity in aqueous solution at a pH value of 8.6 is about 25 times higher than in reverse micelles at W_0 = 20. For lower W_0 values (5 and 10) the plateau of maximum activity is extended to higher pH values in comparison to higher W_0 values (20 and 30). This can be attributed to the fact that small W_0 values produce larger shifts of intramicellar pH (*7*). The selected pH value used in subsequent experiments was 9.6.

Optimizing W_0 for the specific activity greatly depends on the pH, as can be seen in Fig. 1B. In fact the optimum value of W_0 = 10, was only found at pH 10.7. For other pH values, the specific activity increases with

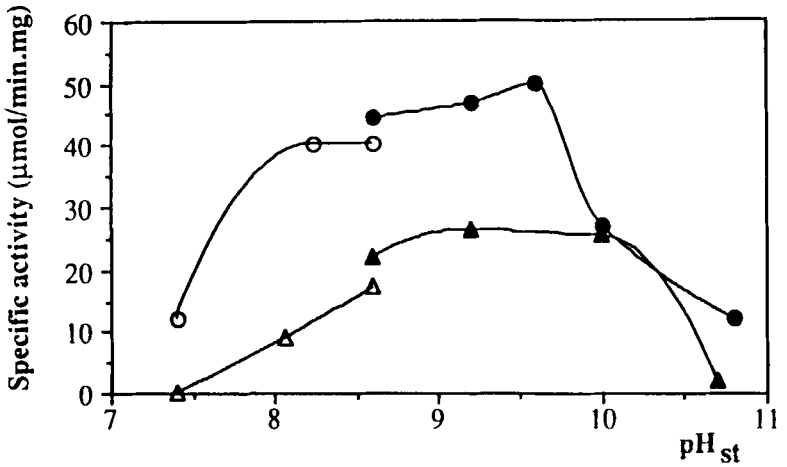

Fig. 2. Effect of pH on triolein hydrolysis by cutinase in reverse micelles of AOT in *iso*-octane at two buffer ionic strenghts: △ ▲ 0.05 *M*; ○ ● 0.2*M*. The empty symbols referred to values obtained with Tris-HCl buffer and the full symbols with carbonate-bicarbonate buffer.

an increase in W_0. The volume of the recombinant cutinase (5) was calculated to be 21205 Å^3 and the inner cavity of the initial empty micelle with W_0 = 10 to be 28722 Å^3 (*8*). This implies that, at pH 10.7 with an optimum W_0, the two volumes, empty micelle and enzyme, are alike, which also seems to be similar to several other enzymes (*9*). For other pH values, the specific activity is greater for bigger micellar volumes. For example, at W_0 = 20 the empty micelle has a volume of 163522 Å^3.

At W_0 = 30 the instability of the system increases, taking a few minutes until a clear solution was reached, hence W_0 = 20 was chosen for further work.

Effect of Buffer Ionic Strength

The effect of the ionic strength of the buffer used for the solubilization of the protein before the microencapsulation, had a marked effect on the specific activity (Fig. 2). This effect was characterized by a twofold increase in specific activity at almost all pH values, although the size of the water pool decreases with higher ionic strengths (*10*).

Since the increase in the specific activity, when the ionic strength is raised from 50 to 200 m*M*, is accompanied by an increase in time needed to microencapsulate the enzyme, subsequent work with 50 m*M* buffer was carried out.

Temperature Profile

The cutinase in reversed micelles of AOT-*iso*-octane is very sensitive to temperature (Fig. 3), and at 35°C cutinase is inactive. This high sensitivity to temperature does not occur in aqueous solution, where the

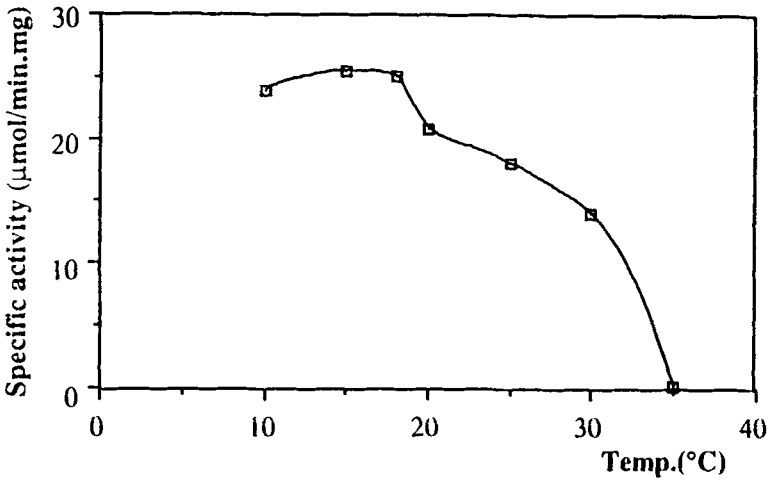

Fig. 3. Effect of temperature on triolein hydrolysis by cutinase in reverse micelles of AOT in *iso*-octane at W_0 = 20, pH 9.6 and 50 m*M* of buffer.

Table 1
Deactivation Rate Coefficients (k_1 and k_2)
and Ratio of Specific Activities (α_1 and α_2) of Cutinase
in Reversed Micelles in AOT-*iso*-Octane and in Aqueous Solution

	k_1, h^{-1}	k_2, h^{-1}	α_1	α_2
W_0 = 5; pH 9.6; T = 30°C	1.62	1.62	0.98	0.46
W_0 = 5; pH 9.6; T = 25°C	3.21	0.29	1.59	0.15
W_0 = 10; pH 9.6; T = 30°C	5.98	0	0	0
W_0 = 20; pH 9.6; T = 30°C	14.87	0	0.07	0
W_0 = 20; pH 9.6; T = 25°C	5.70	5.93	0.58	0.04
W_0 = 20; pH 8.7; T = 30°C	14.01	0	0.07	0
Aq. solution; pH 8.6; T = 30°C	0.146	0.014	0.41	0

recombinant cutinase still works at 85°C (*4*). The microencapsulation of cutinase in the reverse micelles causes its inactivation as shown by the stability studies (*see later*). At W_0 = 20, pH 9.6, and T = 30°C, cutinase has only 7% of its initial activity (Table 1) after 4 min of incubation. At higher temperatures, the interface is more fluid and disordered (*11*), which could cause a faster inactivation, with no activity detected immediately after the microencapsulation. With the selected conditions (W_0 = 20, pH 9.6, and 50 m*M* buffer), it was not possible to microencapsulate the enzyme below 10°C. As the time needed for microencapsulation increases with lower temperatures, 30°C was selected for further work.

Table 2
Apparent Kinetic Constants of Cutinase in Reverse Micelles of AOT-*iso*-Octane for the Triglycerides Hydrolysis with Different Acyl Chain Length[a]

	$k_{cat,app}$/ min^{-1}	$K_{m,app}$, m*M*	$k_{cat.app}/K_{m,app}$/ min^{-1} mM^{-1}
Tricaprylin, C8:0	1089	132	8.2
Trilaurin, C12:0	375	151	2.5
Trimyristin, C14:0	321	151	2.1
Triolein, C18:1,[cis]-9	1630	395	4.1

[a] $K_{m,app}$ was referred to total volume of micellar solution.

Kinetic Constants for Triglyceride Hydrolysis

The kinetic analysis of the hydrolysis was done with four triglycerides with variable acyl chain length: tricaprylin (C8:0), trilaurin (C12:0), trimyristin (C14:0), and triolein (C18:1, [cis]-9). Since the kinetic obeys the classical Michaelis-Menten equation, the kinetic constants $K_{m,app}$ ($K_{m,apparent}$) and $k_{cat,app}$ ($k_{cat,apparent}$) were calculated from a double-reciprocal plot of the cutinase activities (Table 2). The kinetic constants calculated from the double-reciprocal plot are apparent, as they are referred to the total volume of micellar solution and most probably all the catalytic activity is confined to the micellar phase (water solubilized in reverse micelles). In reverse micellar systems $k_{cat,app} = k_{cat,mic}$ (mic refers to micellar phase) but the observed $K_{m,app}$ can differ considerably from the true $K_{m,mic}$ by a factor of 1×10^{-3} to 1×10^{4} (*12*). Thus according to the partition coefficient of the substrate between the organic solvent and the water pool, $K_{m,app}$ can be lower (hydrophilic substrates) or higher (hydrophobic substrates) than $K_{m,mic}$. So the high $K_{m,app}$ values obtained can be explained by the use of a hydrophobic substrate, i.e., poorly soluble in the water pool of reversed micelles, corresponding in fact to $K_{m,mic}$ that can be up to 10,000 times lower.

The second-order rate constant, $k_{cat,app}/K_{m,app}$ decreases with an increase in the acyl chain length, except for triolein, which has one double bond. Despite the fact that this ratio could reflect the differences in hydrophobicity of substrates, between triglycerides with saturated acyl chains the bigger difference is noted in $k_{cat,app}$. This reflects a decrease in specificity with an increase in the acyl chain length. The hydrolytic activity of the native cutinase in aqueous solution on *p*-nitrophenyl esters of fatty acids from C_2 through C_{18}, shows a specificity for esters of short chain fatty acids (*13*). This specificity results mainly from differences in k_{cat}, as also occurred in reverse micelles.

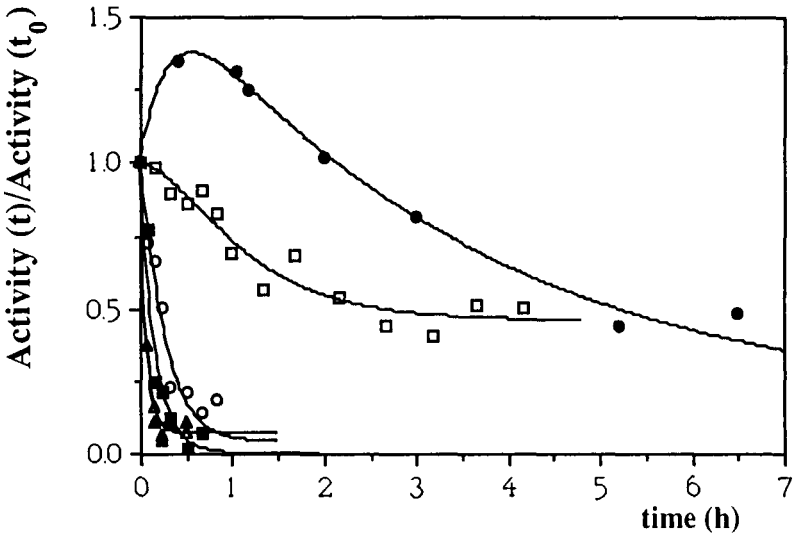

Fig. 4. Deactivation profiles of cutinase in reversed micelles of AOT in *iso*-octane: □ W_0 = 5, pH 9.6, T = 30°C, ● W_0 = 5, pH 9.6, T = 25°C; ■ W_0 = 10, pH 9.6, T = 30°C; ▲ W_0 = 20, pH 9.6, T = 30°C; ○ W_0 = 20, pH 9.6, T = 25°C; △ W_0 = 20, pH 8.7, T = 30°C. (——) Application of Henley and Sadana model (*1*).

Stability of Cutinase in Reversed Micelles

A study of the effect of W_0, pH, and temperature on the stability of recombinant cutinase in reversed micelles was also evaluated. Since the deactivation profile shows an inflection in two conditions tested (Fig. 4) (W_0 = 5, pH 9.6, and T = 30°C and W_0 = 20, pH 9.6, and T = 25°C) and an initial activation at W_0 = 5, pH 9.6, and T = 25°C, the model of Henley and Sadana (*1*) was applied to provide the deactivation rate coefficients (k_1 and k_2) (Table 1). This model incorporates a series-type deactivation with first-order steps:

$$E \xrightarrow{k_1,\ \alpha_1} E_1 \xrightarrow{k_2,\ \alpha_2} E_2$$

where α_1 and α_2 are the ratios of specific activities E_1/E and E_2/E, respectively. The application of the model to the data only allows for two states of enzyme (E_1 and E_2) in the conditions above mentioned, where the stability is higher. It is then possible that a series-type deactivation is also present under the other conditions but the deactivation is so fast that the data do not present sufficient resolution that will fit two states. This is confirmed by the studies mentioned below, all carried out at 25°C, where two states of enzymes are always fitted.

As can be seen from Fig. 4 and Table 1, the stability is low and the deactivation profiles shows a fast inactivation of cutinase after the micro-encapsulation. Preliminary fluorescence spectroscopic studies showed

that this inactivation is owing to the denaturation of cutinase, probably caused by the AOT surfactant (*14*). In addition, the stability decreases significantly with the increase in W_0 (higher k_1). For example, at $W_0 = 20$ and 30°C the activity of cutinase was only 7% ($\alpha_1 = 0.07$) of its initial activity after 4 min ($1/k_1 = 4$ min). At $W_0 = 5$ and the same temperature the activity remains at 98% ($\alpha_1 = 0.98$) after 37 min ($1/k_1 = 37$ min). A second deactivation step to 46% of the initial activity occurs also within 37 min. At 30°C, but in aqueous solution the activity is still 41% of its initial value after 6.8 h, which means that the deactivation is considerably slower than in reverse micelles (Table 1). These results show similarities to the behavior of lipases from *Candida rugosa* and *Rhizopus delemar*, where a pronounced deactivation took place in reversed micelles, especially at W_0 values higher than 3.0 (*15*). However, the degree to which reversed micelles cause deactivation varies significantly with the lipase sources (*15*), and it is much greater in cutinase compared with the other lipases referred to above. A high dependency of stability upon the degree of hydration of the surfactant was also found in lipases of *Candida rugosa* (*16*) and *Chromobacterium viscosum* (*17*). Also, with these two lipases, the stability decreases with higher W_0 values. The water in the interior of reversed micelles is tightly bound at low W_0 values and the mobility is enhanced by increasing W_0, gradually approaching that of bulk water (*18*). This rigid structure of the endomicellar region, particularly at low W_0 values, leads to a more rigid conformation of the protein, namely one characterized by a higher helical content (*19*). The greater conformational constraints imposed on cutinase at low W_0 values could be responsible for the increase in stability. This hypothesis becomes more plausible since the bigger increase in stability ($W_0 = 10 \rightarrow W_0 = 5$) is noted for empty micellar volumes smaller than cutinase (volume of empty micelle = 6292 $Å^3$ at $W_0 = 5$).

The stability behavior with respect to W_0 is opposite to that of the activity that increases with higher W_0, except at pH 10.7. Raising the temperature from 25 to 30°C decreases the stability, and the change of pH from 8.7 to 9.6 has no significant effect.

Interfacial enzymes, such as lipases, can interact with the surface layer of micelles or even penetrate it when its hydrophobicity is high, and may come into contact with the organic solvent (*20*). If this is the case, the organic solvent can be responsible for denaturation of the enzyme. It is known that biocatalytic studies in organic solvents that the higher the log *P* value the higher the biocatalytic rates (*21*). This is because an organic solvent with low polarity is less able to distort the essential water layer that stabilizes the biocatalysts. To check the possibility of contact between cutinase and organic solvent being responsible for the low stability observed, reversed micelles in *n*-dodecane, which has a log *P* of 6.6 (*21*) instead of *iso*-octane (log *P* = 4.5 (*21*)) were also tested. According to log *P* values, a higher stability in reversed micelles with *n*-dodecane would be

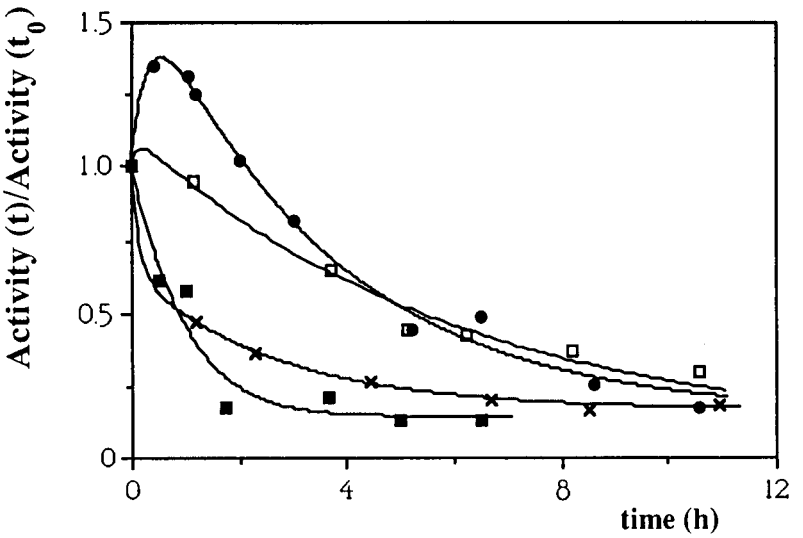

Fig. 5. Deactivation profiles of cutinase in reversed micelles of: ● AOT-*iso*-octane; ■ AOT-*iso*-octane with 100 m*M* oleic acid; □ AOT-*iso*-octane with 100 m*M* glycerol and X AOT-*n*-dodecane at W_0 = 5, pH 9.6, T = 25°C. (——) Application of Henley and Sadana model (*1*).

Table 3
Deactivation Rate Coefficients (k_1 and k_2)
and Ratio of Specific Activities (α_1 and α_2) of Cutinase
in Reversed Micelles of AOT-*iso*-Octane and AOT-*n*-dodecane[a]

	k_1/h^{-1}	k_2/h^{-1}	α_1	α_2
AOT-*n*-dodecane	6.30	0.37	0.61	0.17
AOT-*iso*-octane	3.21	0.29	1.59	0.15
AOT-*iso*-octane, 100 m*M* oleic acid	1.40	1.49	0.46	0.14
AOT-*iso*-octane, 100 m*M* glycerol	1.58	0.16	1	0.06

[a]Conditions: W_0 = 5; pH 9.6; T = 25°C.

expected, but in fact stability is lower (Fig. 5; Table 3), which could be related to different properties of the micellar system when the organic solvent is changed.

Products and/or substrates may increase enzyme stability upon binding at the active site (*22*). The effect of glycerol and oleic acid (hydrolysis products of triolein and substrates for synthesis reactions) on cutinase stability were tested (Fig. 5; Table 3). The initial activation observed when cutinase was incubated without products disappears and there is no significant stabilization in the presence of 100 m*M* of glycerol. There is a

decrease in cutinase stability with oleic acid, which also occurred for the synthesis reaction (*23*). It follows that the stability of cutinase microencapsulated in AOT reverse micelles was not improved by the presence of products and/or substrates.

CONCLUSIONS

The hydrolytic activity of a recombinant cutinase microencapsulated in AOT reverse micelles is lower than in aqueous solution and depends on the physicochemical parameters of the system. Beside the pH and temperature, the water content in the system described by the W_0 parameter (W_0 = [H_2O]/[AOT]) and the ionic strength of the buffer used to solubilize the enzyme before the microencapsulation can raise 10 times the hydrolytic activity on triolein.

The kinetic analysis of triglycerides hydrolysis with variable acyl chain lengths shows a decrease in the specificity with increase in the chain length, except for triolein, which has one double bond. The hydrolytic activity of native cutinase on *p*-nitrophenyl esters of fatty acids shows a similar behavior (*13*).

The stability of the cutinase encapsulated in reverse micelles of AOT-*iso*-octane is lower than in aqueous solution and a fast deactivation of the enzyme is obtained after the microencapsulation. Among the physicochemical parameters of the system, higher temperatures, and W_0 values decrease the stability and raising the pH from 8.7 to 9.6 has no significant effect. The change of solvent (from *iso*-octane to *n*-dodecane) and the presence of triolein hydrolysis products does not prevent the fast deactivation of cutinase that occurs in this system.

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REFERENCES

1. Henley, J. P. and Sadana, A. (1985), *Enz. Microb. Technol.* **7**, 50–60.
2. Laane, C., Hilhorst, R., and Veeger, C. (1987), in *Methods in Enzymology*, Mosbach, K., ed., Academic, New York, vol. 136, pp. 216–219.
3. Kolattudkudy, P. E. (1984), in *Lipases*, Brorström, B. and Brackman, H., eds., Elsevier, Amsterdam, pp. 471–504.

4. Lauwereys, M., de Geus, P., de Meutter, J., Stanssens, P., and Matthyssens, G. (1991), in *Lipases-Structure, Mechanism and Genetic Engineering*, Alberghina, L., Schmid, R. D., and Verger, R., eds., VCH, Weinheim, vol. 16, pp. 243–251.
5. Martinez, C., de Geus, P., Lauwereys, M., Matthyssens, G., and Cambillau, C. (1992), *Nature* **356,** 615–618.
6. Lowry, R. R. and Tinsley, J. T. (1976), *J. Am. Chem. Soc.* **53,** 470–472.
7. Shield, J. W., Fergunson, H. D., Bommarius, A. S., and Hatton, T. A. (1986), *Ind. Eng. Chem. Fundam.* **25,** 603–612.
8. Eicke, H.-F. and Rahak, J. (1976), *Helvetica Chim. Acta* **59,** 2883–2891.
9. Martinek, K., Klyachko, N. L., Kabanov, A. V., Khmelnitsky, Y. L., and Levashov, A. V. (1989), *Biochim. Biophys. Acta* **981,** 161–172.
10. Sheu, E., Göklen, K. E., Hatton, T. A., and Chen, S. H. (1986), *Biotechnol. Prog.* **2,** 175.
11. Hayes, D. G. and Gulari, E. (1991), *Biotechnol. Bioeng.* **38,** 507–517.
12. Martinek, K., Levashov, A. V., Klyachko, N. L., Pantin, V. I., and Berezin, I. V. (1981), *Biochim. Biophys. Acta* **657,** 277–294.
13. Purdy, R. E. and Kolattudkudy, P. E. (1975), *Biochemistry* **14,** 2832–2840.
14. Melo, E. P., Costa, S. M. B., and Cabral, J. M. S. (1992), in *CEC International Workshop in Lipase: Structure, Mechanism and Genetic Engineering*, Capry, Italy, p. 64.
15. Hayes, D. G. and Gulari, E. (1990), *Biotechnol. Bioeng.* **35,** 793–801.
16. Han, D. and Rhee, J. S. (1986), *Biotechnol. Bioeng.* **28,** 1250–1255.
17. Fletcher, P. D. I., Robinson, B. H., Freedman, R. B., and Oldfield, C. (1985), *J. Chem. Soc. Faraday Trans. I* **81,** 2667–2679.
18. Wong, M., Thomas, J. K., and Nowak, T. (1977), *J. Am. Chem. Soc.* **99,** 4730–4736.
19. Steinmann, B., Jäckle, H., and Luisi, P. L. (1986), *Biopolymers* **25,** 1133–1156.
20. Martinek, K., Levashov, A. V., Khmelnitski, Yu. L., Klyachko, N. L., and Berezin, I. V. (1982), *Science* **218,** 889–891.
21. Laane, C., Boeren, S., Hilhorst, R., and Veeger, C. (1987), in *Biocatalysis in Organic Media*, Laane, C., Tramper, J., and Lilly, M. D., eds., Elsevier, Amsterdam, pp. 65–84.
22. Villaume, I., Thomas, D., and Legoy, M. D. (1990), *Enz. Microb. Technol.* **12,** 506–509.
23. Sebastião, M. J., Cabral, J. M. S., and Aires-Barros, M. R. (1993), *Biotechnol. Bioeng.* **42,** 326–332.