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Superactivity of Peroxidase Solubilized in Reversed Micellar Systems

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ABSTRACT

Vaccinium mirtyllus peroxidase solubilized in reversed micelles was used for the oxidation of guaiacol. Some relevant parameters for the enzymatic activity, such as pH, w_0 (molar ratio water/surfactant), surfactant type and concentration, and cosurfactant concentration, were investigated. The peroxidase showed higher activities in reversed micelles than in aqueous solution. The stability of the peroxidase in reversed micelles was also studied, namely, the effect of w_0 and temperature on enzyme deactivation. The peroxidase displayed higher stabilities in CTAB/hexanol in isooctane reversed micelles, with half-life times higher than 500 h.

Index Entries: *Vaccinium mirtyllus* peroxidase; reversed micelles; superactivity; stability.

INTRODUCTION

Peroxidase (EC 1.11.1.7) catalyzes the oxidation of a wide variety of compounds, such as phenols, aromatic amines, reduced aromatic sulfides, and other reducing equivalents, including highly substituted methoxybenzenes (*1,2*) and nonaromatic compounds (*3*). Usually, the reaction mechanism of peroxidase is characterized by an initial two-electron oxidation of the native ferric enzyme to an oxidized intermediate in the presence

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of hydrogen peroxide. The regeneration of the native peroxidase is accomplished by two sequential one-electron reductions through a partially oxidized intermediate (*4*).

When solubilized in nonaqueous medium, enzymes alter their native, aqueous-based structure and function (*5*) usually with a concomitant reduction of their catalytic activity. In reversed micellar systems, enzymes are stabilized in the organic solvent by a surfactant layer and thus protected from the denaturing effect of the organic phase. These systems allow an easy control of the water concentration, and make possible the solubilization of both hydrophilic and hydrophobic substrates or products.

Some authors have studied the behavior of peroxidase in reversed micelles founding significant increase of the activity of this enzyme, when compared with aqueous activity (*6–8*). This superactivity has been attributed to the increased conformational rigidity of the enzyme (*9*), the reduction of substrate inhibition (*10*), and the decrease of polarity in the enzyme catalytic site (*11,12*).

In this article, the activity and stability of an extracellular peroxidase from *Vaccinium mirtyllus* cell suspension cultures solubilized in reversed micelles were studied. Three surfactants differing in their net charge, sodium dioctylsulfosuccinate (AOT) (anionic), hexadecyltrimethylammonium bromide (CTAB) (cationic), and phosphatidylcoline (PHDC) (nonionic) were used to form reversed micelles in isooctane. In the case of CTAB, a cosurfactant (hexanol) essential for the stabilization of reversed micelles was introduced. The effect of the surfactant charge on the activity and stability of the peroxidase was evaluated. Other parameters, such as the cosurfactant concentration, the amount of solubilized water, and pH, were also studied.

MATERIALS AND METHODS

Enzyme

The peroxidase used was obtained from the culture medium of *V. mirtyllus* suspension cell cultures. This peroxidase has a mol wt of 35,000 Dalton. The production and partial characterization of this enzyme were described by Melo et al. (*13*).

Chemicals

Sodium dioctylsulfosuccinate (AOT) (99% pure), hexadecyltrimethylammonium bromide (CTAB) (99% pure), and L-α-Phosphatidylcholine (Type XII-E) (PHDC) (64% pure) was purchased from Sigma (St. Louis, MO). Isooctane (99.5% pure) was from Riedel-de Haen, and guaiacol (> 99% pure) and hydrogen peroxide (30.0% w/v) were from BDH Chemicals (Poole, England). 1-Hexanol (98% pure) and salts (analytical reagent grade) were from Merck (Darmstadt, West Germany).

Peroxidase Activity Assays

Peroxidase Activity in Aqueous Solution

Peroxidase activity was assayed using a modification of the method described by Chance and Machly (*14*). Ten microliters of a stock enzyme solution (30 mg/L) and 50 μL of guaiacol solution (500 m*M* in ethanol) were added to a test tube containing 5 mL of buffer solution (25 m*M* phosphate buffer, pH 6.5). After vortex mixing for 1 min, 1 mL of this solution was then added into a 1-mL cuvet and the reaction was started with the addition of 2 μL of hydrogen peroxide solution (250 m*M*). The reactions were followed for 1 min by reading the increase in absorbance at 470 nm. The extinction molar coefficient of the oxidation product, $\epsilon = 26.6$/cm m*M*, was used for calculation of the activities, in aqueous and reversed micellar solutions.

Peroxidase Activity in AOT and CTAB Reversed Micelles

Reversed micelles of AOT and CTAB containing the peroxidase (6×10^{-2} mg/L) were obtained by injecting appropriate amounts of a peroxidase solution in aqueous buffer (25 m*M* phosphate buffer, pH 6.5) into 5 mL of AOT (100 m*M*) in isooctane, or CTAB (100 m*M*)/11% (v/v) hexanol in isooctane. After vortex mixing for few seconds, clear micellar solutions were obtained. Two hundred microliters of guaiacol solution (500 m*M* in isooctane) were then added to the reversed micelles. After waiting for 5 min, 1 mL of this solution was added into a 1-mL cuvet, and the reaction was started with the addition of 2 μL of hydrogen peroxide solution (250 m*M*). The hydrogen peroxide concentrations in the water pool of reversed micelles are between 3.5 and 34.7 m*M*, depending on the water content of the micellar solutions. The reactions were followed for 1 min by reading the increase in absorbance at 470 nm. The water content of the micellar solutions was quantified using a Mettler DL18 Karl Fisher titrator; w_o was defined as the molar ratio [water]/[surfactant].

Peroxidase Activity in PHDC Reversed Micelles

Reversed micelles of PHDC containing the peroxidase (6×10^{-2} mg/L) were obtained by injecting appropriate amounts of a solution of peroxidase in 25 m*M* phosphate buffer, pH 6.5, into 5 mL of PHDC/hexanol (3% v/v) in isooctane. After vortex mixing, 10 μL of hydrogen peroxide solution (250 m*M*) were added to the mixture. To obtain a clear solution, the mixture was sonicated for 15 s (frequency 20 kHz). One milliliter of this solution was then added into a 1-mL cuvet, and the reaction was started with the addition of 40 mL of guaiacol solution (500 m*M* in isooctane). The reactions were followed for 1 min by reading the increase in absorbance at 470 nm.

Peroxidase Stability Assays

Peroxidase (6×10^{-2} mg/L) solubilized in reversed micelles was incubated in capped test tubes in a thermostated bath, without stirring, at

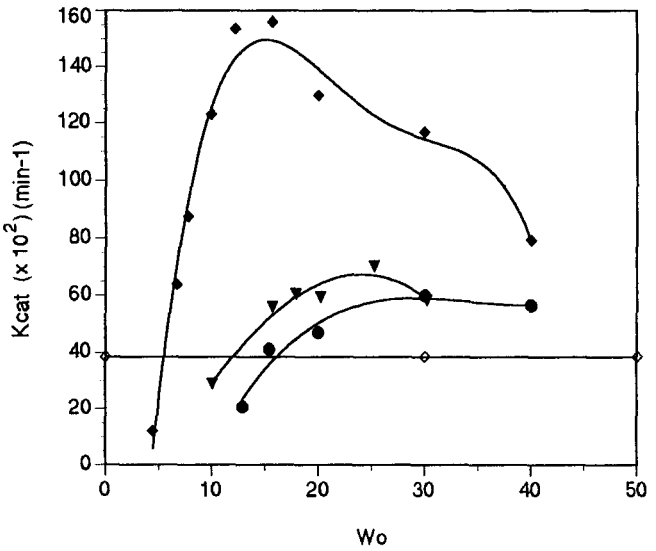

Fig. 1. Effect of w_o on the catalytic constant (k_{cat}) of peroxidase solubilized in AOT (100 m*M*)/isooctane (◆), CTAB (100 m*M*)/hexanol (11% v/v)/isooctane (▼), and PHDC (39 m*M*)/hexanol (3% v/v)/isooctane (●) reversed micelles and in aqueous solution, at 25°C and pH 6.5 (◇).

25, 35, and 50°C for AOT (100 m*M*) and CTAB (100 m*M*)/hexanol (11% v/v) and 20°C for PHDC (39 m*M*)/hexanol (3% v/v). Independent reaction systems were prepared for the different incubation times using w_o = 10, 16, and 25 for AOT and CTAB and w_o = 10, 15, and 30 for PHDC. In AOT and CTAB systems, the peroxidase was incubated in the presence of the guaiacol and in PHDC with the hydrogen peroxide. Peroxidase activity was measured as described above.

RESULTS AND DISCUSSION

Effect of the Type of Surfactant and Amount of Water on Peroxidase Activity

The catalytic activity of peroxidase in aqueous solution and in AOT, CTAB and PHDC reversed micelles for different w_o values is shown in Fig. 1. The value of the catalytic constant, k_{cat}, was calculated from the maximum rate of the process ($V = k_{cat}$ [E]$_o$) corresponding to the conditions of saturation, for both substrates, guaiacol and hydrogen peroxide.

A superactivity at the optimum w_o value was observed for the three tested systems. Catalytic constants of 1.5 to 4.5-fold higher than the value found for the free enzyme in aqueous solution were obtained. The highest k_{cat} values were obtained for the peroxidase solubilized in reverse micelles of AOT/isooctane.

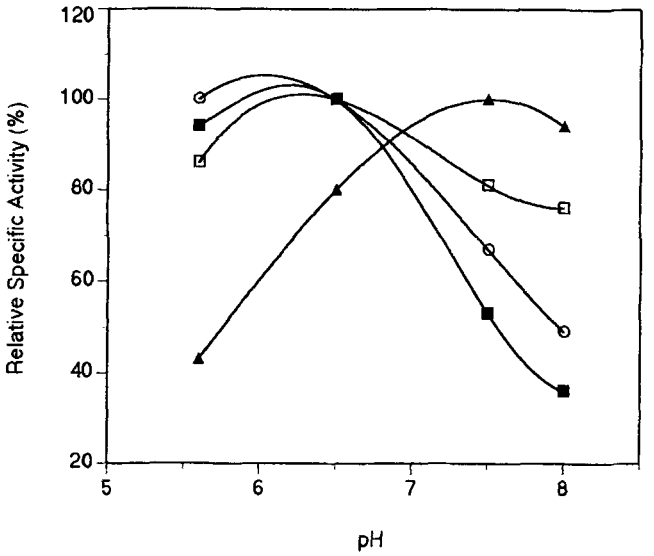

Fig. 2. Effect of pH on the specific activity of peroxidase solubilized in AOT (100 m*M*)/isooctane (○), CTAB (100 m*M*)/hexanol (11% v/v)/isooctane (▲), and PHDC (39 m*M*)/hexanol (3% v/v)/isooctane (□) reversed micelles and in aqueous solution (■), at 25°C and w_o = 16.

The superactivity of peroxidase in the AOT system was also described by Martinek and coworkers (*15*). The superactivity was attributed to a more rigid conformation of the enzyme into the micelle (*9*), and to the reduction of substrate and product inhibitions (*15*).

The optimum of k_{cat} in AOT/isooctane reversed micelles was found at w_o = 14, which is in accordance with the value reported by Martinek et al. (*16*). At this w_o, the diameter of the inner cavity of the reversed micelle is approximately equal to 50 Å (*17*), which is near the calculated value for the diameter of the peroxidase molecule (*18*). The optimum of k_{cat} shifts to higher values of w_o for CTAB and PHDC reversed micelles, being w_o = 25 and w_o = 30, respectively.

Effect of pH on Peroxidase Activity

In an aqueous system, the optimum pH value for the oxidation of guaiacol by peroxidase is 6.2 (Fig. 2). For the solubilized peroxidase (w_o = 16), three different behaviors were observed. In the AOT/isooctane system, a slight shift of the optimum pH to a lower value was found, whereas in CTAB/hexanol/isooctane system, the optimum pH was shifted in the opposite direction. In the PHDC/hexanol/isooctane system, the optimum pH is similar to that obtained in aqueous solution (Fig. 2).

The results obtained can be explained by a proton gradient in the water pool of reversed micelles induced by the surfactant charge. Experimental results showed complete inactivation of this enzyme in the presence of isooctane. The catalytic site of most studied peroxidases is buried inside

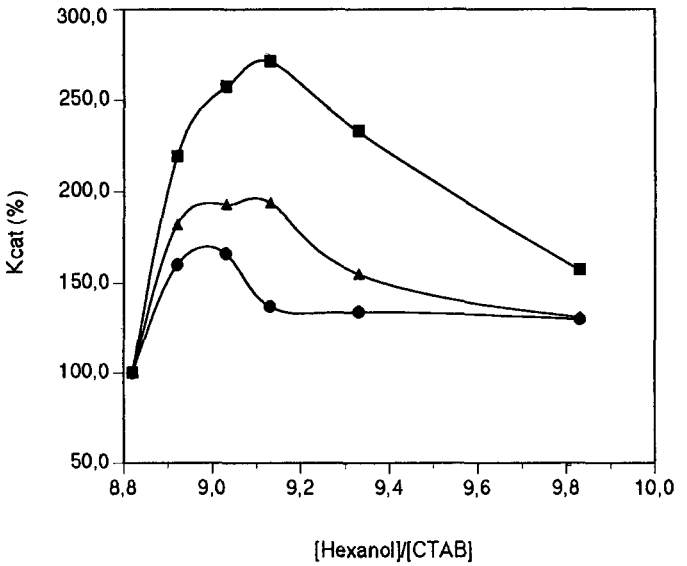

Fig. 3. Effect of the molar ratio hexanol/CTAB on the catalytic constant (% of k_{cat} relatively to k_{cat} for a ratio hexanol/CTAB of 8.8) of peroxidase solubilized in CTAB (100 m*M*)/hexanol/isooctane at w_o = 10 (■), w_o = 16 (●), and w_o = 25 (▲), at 25°C and pH 6.5.

the peptide molecule (*19*) and is, therefore, away from the inner micelle surface when entrapped in reversed micelles. Under this condition, a shift of the optimum pH to lower values is expected for anionic surfactants, owing to a decrease in proton concentration in the inner core of the water pool; a shift in the opposite direction should also be observed for cationic surfactants. For nonionic surfactants, the optimum pH should be identical to that in the bulk water, since there is no gradient of protons inside the water pool.

Effect of Cosurfactant Concentration on Peroxidase Activity

Figure 3 shows the effect of the molar ratio [hexanol]/[surfactant] on the activity of the peroxidase in CTAB (100 m*M*)/hexanol/isooctane reverse micelles. An increase in the k_{cat} was observed when the ratio [hexanol]/[surfactant] was increased, with a maximum around [hexanol]/[surfactant] = 9, at the w_o values studied. The highest k_{cat} was obtained at w_o = 25. Under this condition, the value obtained for the k_{cat} (136 × 10^2 min^{-1}) was near the optimum observed for the AOT/isooctane system (156 × 10^2 min^{-1} at w_o = 10). Increasing the alcohol concentration in a microemulsion of cationic surfactant leads to an increase in interface elasticity and micelle dimension (*20*), which can explain the increase in k_{cat} found for ratios slightly above the standard ratio used (8.8 = 11% v/v hexanol). However, when the interface elasticity is too high, a decrease in the micelle dimension was observed (*20*), which may explain the reduction in k_{cat} at higher

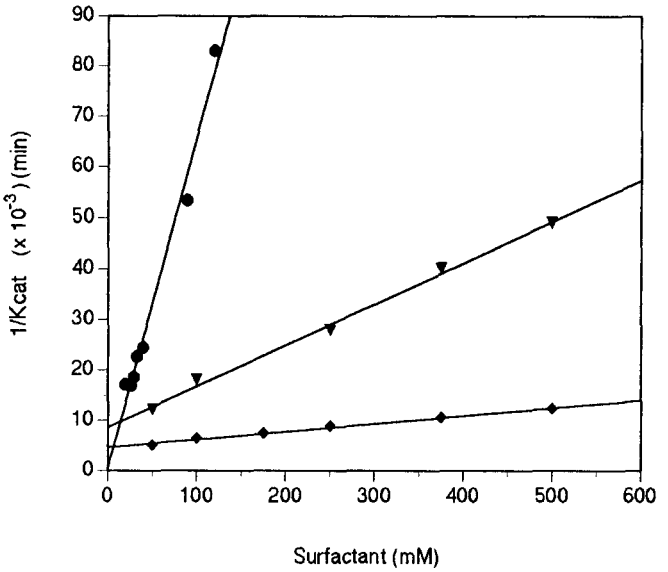

Fig. 4. Effect of AOT (◆), CTAB (▼), and PHDC (●) concentration on the catalytic constant of peroxidase at 25°C, pH 6.5, and w_o = 16.

ratio values. In PHDC reversed micelles, no appreciable effect on the peroxidase catalytic activity was observed by changing the hexanol/surfactant ratio.

Effect of Surfactant Concentration on Peroxidase Activity

The k_{cat} of solubilized peroxidase in reversed micelles depends strongly of the surfactant concentration as shown in Fig. 4. Martinek et al. (*15,16*) observed the same behavior and suggest that this was the result of the presence of anchoring groups of a different nature capable of interacting with micellar membranes.

A linear correlation between k_{cat} and surfactant concentration was found, for the three different systems studied, at a constant enzyme concentration and w_o = 16 (Fig. 4). The following equation was obtained:

$$1 / k_{cat} = 1 / k_{cat}(0) + B \times [\text{surfactant}] \quad (1)$$

in which $1/k_{cat}$ (0) represents, at the limit, the k_{cat} without interaction effects between the surfactant and the enzyme, and B is a constant characteristic of the kind of surfactant used.

The k_{cat} (0) values obtained for the three surfactants were: $k_{cat}(0)_{AOT}$ = 217; $k_{cat}\cdot(0)_{CTAB}$ = 116; and $k_{cat}(0)_{PHDC}$ = 1850 min^{-1}. The values obtained for k_{cat} (0) probably reflect the properties of the water pool that are different for the three surfactants tested. The B values obtained were B_{AOT} = 1.57 × 10^{-5}; B_{CTAB} = 8.13 × 10^{-5}; and B_{PHDC} = 6.54 × 10^{-4} min/m*M*, showing that the PHDC system is the most affected by the surfactant concentration, followed by CTAB and AOT systems. This suggests a higher degree of interaction between the PHDC and the peroxidase.

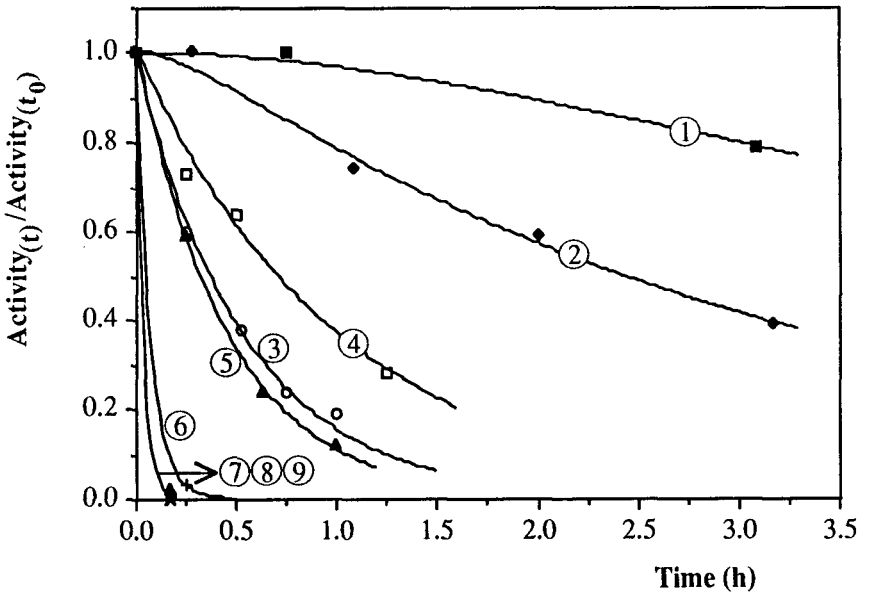

Fig. 5. Experimental deactivation data and deactivation profiles (continuous curves) obtained from the application of Henley and Sadana model of peroxidase in AOT (100 m*M*)/isooctane reversed micelles: (1) T = 25°C, w_o = 30; (2) T = 25°C, w_o = 16; (3) T = 25°C, w_o = 8; (4) T = 35°C, w_o = 30; (5) T = 35°C, w_o = 16; (6) T = 35°C, w_o = 8; (7) T = 50°C, w_o = 30; (8) T = 50°C, w_o = 16; (9) T = 50°C, w_o = 8.

Stability Studies

Stability studies of the peroxidase in reversed micelles of AOT, CTAB, and PHDC, were carried out at different temperatures and w_o values. The model of Henley and Sadana (*21*) was applied to the experimental values obtained for the enzyme deactivation. This model incorporates a series-type enzyme activation with two steps: the first involving an active enzyme precursor and a final enzyme state with possible nonzero activity.

The fit of the curves obtained is shown in Figs. 5, 6, 7, and 8. This model fits reasonably well with the deactivation data found for the enzyme activity in reversed micelles and in the aqueous solution. The half-life times were calculated from the deactivation profiles (Table 1).

The peroxidase stability decreased with increasing temperature for reversed micellar systems and aqueous solution. In the AOT and PHDC systems, the half-life times obtained were much lower than in aqueous solution, for all the tested temperatures. The peroxidase stability obtained in these two systems depends on the w_o used. A decrease in the half-life times was obtained for lower w_o values (Table 1).

Higher stabilities were obtained in the CTAB reversed micelles system. At the temperature of 25°C and w_o of 16 and 25, the stability was higher than in aqueous solution. After 500 h, the peroxidase activity was 180%

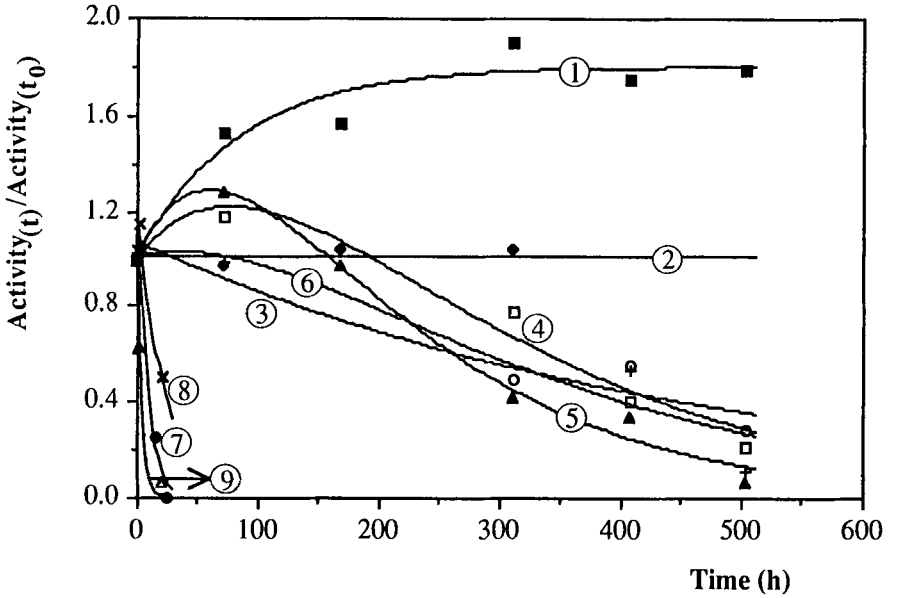

Fig. 6. Experimental deactivation data and deactivation profiles (continuous curves) obtained from the application of Henley and Sadana model of peroxidase in CTAB (100 m*M*)/hexanol (11% v/v)/isooctane reversed micelles: (1) T = 25°C, w_o = 25; (2) T = 25°C, w_o = 16; (3) T = 25°C, w_o = 10; (4) T = 35°C, w_o = 25; (5) T = 35°C, w_o = 16; (6) T = 35°C, w_o = 10; (7) T = 50°C, w_o = 25; (8) T = 50°C, w_o = 16; (9) T = 50°C, w_o = 10.

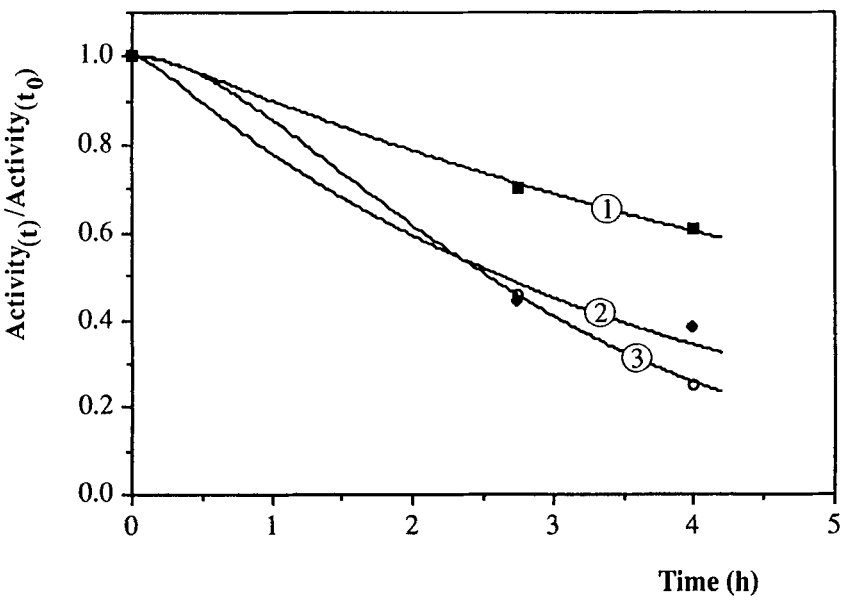

Fig. 7. Experimental deactivation data and deactivation profiles (continuous curves) obtained from the application of Henley and Sadana model of peroxidase in PHDC (39 m*M*)/hexanol (3% v/v)/isooctane reversed micelles: (1) T = 20°C, w_o = 30; (2) T = 20°C, w_o = 15; (3) T = 20°C, w_o = 10.

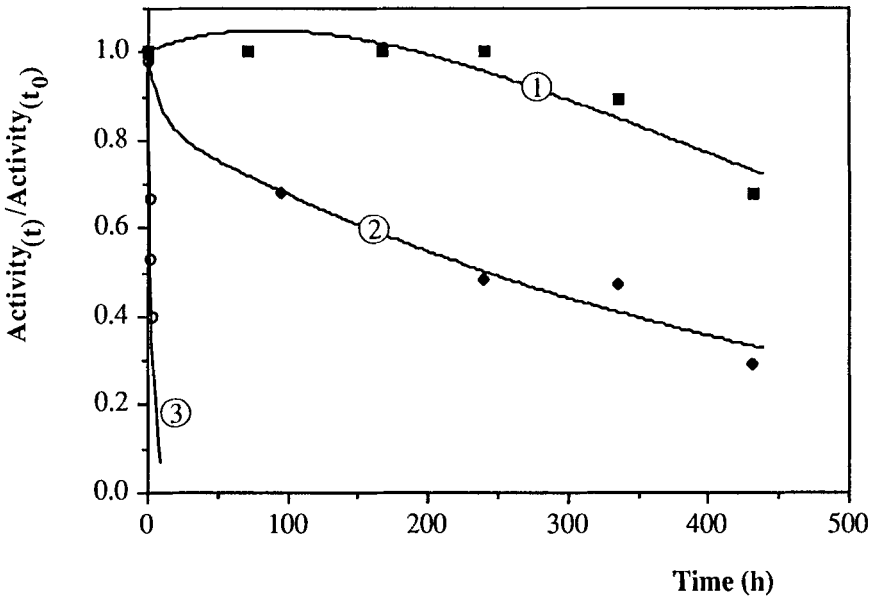

Fig. 8. Experimental deactivation data and deactivation profiles (continuous curves) obtained from the application of Henley and Sadana model of peroxidase in aqueous solution: (1) T = 25°C; (2) T = 35°C; (3) T = 50°C.

for w_o = 25 and 100% for w_o = 16 (referred to the initial activity), whereas the half-life time in aqueous solution was 633 h. At 25°C, a decrease in the w_o from 25 to 10 led to a lower stability, with a half-life time of 345 h. At 35 and 50°C, the stability was less affected by the w_o.

The low stability of peroxidase solubilized in PHDC reversed micelles is probably owing to the oxidation of the phospholipid molecules by the hydrogen peroxide, since the peroxidase was incubated in the presence of this substrate. The oxidation of the surfactant layer could lead to peroxidase denaturation because of the contact with the organic solvent.

The stability of peroxidases in AOT systems was also low, decreasing for lower w_o values, which suggests an inhibitory effect of the surfactant. As the peroxidase is solubilized in the water pool, the decreased stability for lower w_o values is probably the result of the proximity of the enzyme to the micellar interface.

Among the systems studied, CTAB was shown to be the most appropriate to stabilize the peroxidase activity, namely at 50°C, where an increase in the half-life time ($t_{1/2}$ = 20 h) of eightfold was obtained when compared with aqueous solution ($t_{1/2}$ = 2.5 h).

CONCLUSIONS

V. mirtyllus peroxidase showed a superactivity against guaiacol when solubilized in reversed micelles of ionic and neutral surfactants, with

Table 1
Half-Life Times ($t_{1/2}$) for Peroxidase in Aqueous Solution and Solubilized in Reversed Micelles of AOT (100 m*M*), CTAB (100 m*M*)/hexanol (11%), and PHDC (39 m*M*)/hexanol (3%)[a]

Temperature, °C	$t_{1/2}$, h, Aqueous solution	w_o	$t_{1/2}$, h AOT	$t_{1/2}$, h CTAB	$t_{1/2}$, h PHDC
		10			2.5
20	—	15	—	—	2.6
		30			5.4
		8	0.4	—	
		10	—	345	
25	633	16	2.4	>500	—
		25	—	>500	
		30	6.1	—	
		8	0.05	—	
		10	—	339	
35	240	16	0.32	292	—
		25	—	381	
		30	0.71	—	
		8	0.03	—	
		10	—	3.3	
50	2.5	16	0.03	20	—
		25	—	10	
		30	0.03	—	

[a]Data obtained from deactivation profiles of Figs. 5, 6, 7, and 8.

activities 1.5-fold to fourfold higher than in aqueous solution. The highest activity was obtained with the enzyme solubilized in reversed micelles of AOT. However, a low stability was found for this system, with half-life times between 20 min and 6 h.

Higher stabilities were obtained for the peroxidase solubilized in CTAB/hexanol/isooctane reversed micelles. By changing the ratio hexanol/CTAB, the w_o, and the surfactant concentration, it was possible to obtain peroxidase activities near those found with the AOT system. At these conditions (hexanol/CTAB = 9.0, w_o = 25, and [surfactant] = 50 m*M*), the CTAB/hexanol system is a good alternative for guaiacol oxidation by peroxidase solubilized in reversed micelles.

REFERENCES

1. Ryu, K. and Dordick, J. S. (1992), *Biochemistry* **31,** 2588–2598.
2. Tanrog, A., Dorris, M. L., and Guziec, F. S. (1992), *Anal. Biochem.* **205,** 271–277.

3. Halliwell, B. and de Rycker, J. (1988), *Photochem. Photobiol.* **28,** 757–763.
4. Ryu, K. and Dordick, J. S. (1992), *Biochemistry* **31(9),** 2588–2598.
5. Singer, S. J. (1962), *Adv. Protein. Chem.* **17,** 1–68.
6. Martinek, K., Levashov, A. V., Khmelnitski, Yu. L., Klyachko, N. L., and Berezin, I. V. (1982), *Science* (Washington, DC) **218,** 889–891.
7. Klyachko, N. L., Levashov, A. V., and Martinek, K. (1984), *Mol. Biol.* **18,** 1019–1032.
8. Martinek, K., Khmelnitski, Yu. L., Levashov, A. V., and Berezin, I. V. (1982), *Dokl. Akad. NanK. SSSR* **263,** 737–741.
9. Barbaric, S. and Luisi, P. L. (1981), *J. Am. Chem. Soc.* **103,** 4239–4244.
10. Martinek, K., Levashov, A. V., Klyachko, N. L., Pautin, V. I., and Berezin, I. V. (1981), *Biochem. Biophys. Acta* **657,** 277–294.
11. Belonogova, O. V., Likhtenshtein, G. I., Levashov, A. V., Khmelnitski, Y. L., Klyachko, N. L., and Martinek, K. (1983), *Biokhimiya* (Moskow) **48,** 379–386.
12. Shield, J. W., Ferguson, H. D., Bommarius, A. S., and Hatton, T. A. (1986), *Ind. Eng. Chem. Fundam.* **25(4),** 603–612.
13. Melo, N., Gomes, A., Cabral, J. M. S., and Fevereiro, P. (1995), *Plant Sci.* **106,** 177–184.
14. Chance, B. and Machly, A. C. (1964), *Methods Enzymol.* **2,** 764–775.
15. Martinek, K., Klyachko, N. L., Kabanov, A. V., Khmelnitsky, Yu. L., and Levashov, A. V. (1989), *Biochim. Biophys. Acta* **981,** 161–172.
16. Martinek, K., Klyachko, N. L., Levashov, A. V., and Berezin, I. V. (1983), *Dokl. Akad. Nauk. SSSR* **269(2),** 491–493.
17. Levashov, A. V., Khmelnitsky, Yu. L., Klyachko, N. L. et al. (1982), *J. Colloid. Interface Sci.* **88(2),** 444–457.
18. Clementi, F. and Palade, C. E. (1969), *J. Cell. Biol.* **41,** 33–58.
19. Welinder, K. G. (1985), *Eur. J. Biochem.* **151,** 497–550.
20. Leung, R. and Shah, D. O. (1987), *J. Coll. Interf. Sci.* **120,** 330–344.
21. Henley, J. P. and Sadana, A. (1985), *Enzyme Microb. Technol.* **7,** 50–60.