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Micellar catalysis of polyphenol oxidase in AOT/cyclohexane

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Abstract

The catalytic behaviour of mushroom polyphenol oxidase has been studied in dioctylsulphosuccinate (AOT)/cyclohexane reverse micelles. The steady-state conditions were accomplished up to 20 min and 17 μg protein in the assay towards 4-methylcatechol and no loss of specific activity was observed relative to aqueous medium. The pH activity profile of the enzyme was kept in reverse micelles as in water, showing a plateau between 5 and 6.5. The stability of polyphenol oxidase to pH was also studied and about 20% inactivation was found in reverse micelles relative to aqueous medium at neutral pHs. Moreover there was a decrease of stability at acidic pHs. The optimum W_o obtained was 20 and the enzyme was nearly independent of the surfactant concentration at constant W_o.

Kinetic studies of polyphenol oxidase towards several substrates showed that the substrate inhibition by *p*-cresol and 4-methylcatechol observed in buffer was not kept in AOT/cyclohexane reverse micelles. Moreover, the K_m increased and the catalytic efficiency (V/K_m) of the enzyme decreased as the hydrophobicity of substrates was increased.

Keywords: Polyphenol oxidase; Tyrosinase; Reverse micelles; Kinetics; pH stability

1. Introduction

Polyphenol oxidase (EC 1.14.18.1), also known as tyrosinase, is the only enzyme known to catalyze the regioselective aerobic oxidation of monophenols to *o*-diphenols, which are then dehydrogenated to give *o*-quinones as the final product. These *o*-quinones are unstable compounds in aqueous reaction media and so they can undergo a set of chemical reactions that lead to more stable compounds [1]. It is advisable to carry the enzymatic reaction in an apolar medium to avoid quinone polymerization. To achieve this goal, several systems with immobilized polyphenol oxidase catalyzing in an organic solvent with low water content were developed [2]. Also, several supports were assayed in organic media to immobilize the enzyme [3], and the effect of the temperature on the stability [4] and activity of polyphenol oxidase [5] was checked. Moreover, microheterogeneous systems with the enzyme solved in reverse micelles of Brij 96/cyclohexane [6] or dioctylsulphosuccinate (AOT)/isooctane [7] were developed and the effect of substrate partition on the kinetics of the enzyme in reverse micelles was studied [8].

Among other applications, reverse micelles are nowadays being employed in biosensors construction either as vehicle to carry environmental contaminants, such as phenols [9], or as systems carrying the

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enzyme and hydrating it while membranes are formed to act as probes [10]. The aim of our present work is to characterize mushroom polyphenol oxidase in reverse micelles AOT/cyclohexane in order to achieve optimal operation conditions with regard to aqueous media as well as to study its kinetic behaviour towards several substrates.

2. Experimental

2.1. Materials

Mushroom polyphenol oxidase was purchased from Sigma (Spain) with a catechol oxidase activity of 400,000–500,000 units mg^{-1} solid and used without further purification. Phenol and *p*-cresol were from Merck (Darmstadt, Germany). Cyclohexane was from Fluka (Switzerland). AOT was purchased as the sodium salt and employed without further purification from Sigma as well as 4-methylcatechol, catechol, L-DOPA and L-tyrosine whereas 4-*t*-butylphenol and 4-*t*-butylcatechol were purchased from Aldrich (Spain). Other reagents were of analytical grade from Merck.

2.2. Methods

2.2.1. Measurements of the activity

The standard aqueous enzymatic assay was carried out with 8–12 μg protein in a final volume of 1 ml of 25 mM phosphate buffer, pH 6.5, 2 mM in 4-methylcatechol, in a water bath (Selecta) for 5 min at 30°C. After cooling in ice, the absorbance of the 4-methylquinones formed was recorded at 400 nm in a DU-70 spectrophotometer (Beckmann). The molar absorption coefficient was determined previously by mixing 0.5 ml of a solution, either 0.2, 0.1 or 0.05 mM of 4-methylcatechol, in distilled water and 0.5 ml of a solution saturated with sodium periodate.

The standard micellar enzymatic assay was carried out in 25-ml capped flasks in an orbital incubator SI 50 (Stuart Scientific) at 200 rpm for 10 min at 45°C. Between 8–12 μg protein dissolved in 15μl of 25 mM sodium phosphate buffer, pH 6.5, were added to previously formed reverse micelles containing 5 ml of 50 mM AOT/cyclohexane, 2.4 mM in 4-methylcatechol and 75 μl of the same buffer. The final W_o in the assay was 20. After cooling in water/ice, the absorbance was recorded at 378 nm. The molar absorption coefficient of 4-methylquinones was determined with reverse micelles containing 0.2, 0.1 and 0.05 mM of 4-methylcatechol and a saturated solution of sodium periodate in distilled water, following the procedure of Bru et al. [7]. The AOT to form the micelles was first suspended in distilled water, split into Pyrex tubes and freeze-dried following the procedure previously described [11].

The molar absorption coefficient for catechol and 4-*t*-butylcatechol were determined in aqueous and micellar medium as described above and the molar absorption coefficient of L-DOPA was determined only in aqueous medium.

2.2.2. Analytical

The water content of cyclohexane and of micellar solutions was measured by titrating 0.4 ml or 0.1 ml sample, respectively, in a DL 18 Karl-Fischer titrator (Mettler).

The free acid content of AOT was determined by titrating 20 ml of 25 mM AOT solution in methanol/water (1:1, v/v) with 25 mM NaOH in the presence of thymolphthalein.

The protein content was measured according to the Lowry method with bovine serum albumin as standard [12].

3. Results and discussion

The titration of commercial cyclohexane gave 0.0389% water content (w/w). AOT showed a single peak at 230 nm when its absorption spectrum was recorded in cyclohexane, and the AOT titration gave a free acid content of 1.3%. The molar absorption coefficient determined for catechol was 760 at 388 nm in water and 430 at 372 nm in micellar medium. The coefficient for 4-methylcatechol was 1350 at 400 nm in water and 2755 at 378 nm in reverse micelles. The coefficient for 4-*t*-butylcatechol was 822 at 400 nm in water and 3618 at

366 nm in reverse micelles. The coefficient for L-DOPA was 1450 at 478 nm in water.

The activity of polyphenol oxidase towards 4-methylcatechol in reverse micelles ($W_o = 20$) was linear up to 20 min of assay (not shown) which contrasts with the short linearity time found in aqueous medium (up to 5 min). It seems that the quinones produced in the catalytic reaction which polymerize quickly in water, are prevented to do so in reverse micellar medium due to their release into the organic solvent. As a consequence, in organic media they are stable for a longer time making its measurement easy.

3.1. Activity vs. protein

The activity of polyphenol oxidase towards 4-methylcatechol was assayed in buffer and in reverse micelles ($W_o = 20$) as a function of the amount of protein and results are depicted in Fig. 1. We see that linearity is maintained up to 11 μg/ml in buffer, which corresponds to 11 μg/ml in the assay. In reverse micelles, linearity is maintained up to 17 μg suggesting that the increase in the addition of protein is followed by an increase in the number of enzyme-containing reversed micelles and hence by the overall catalytic activity of the system. The reaction, up to 17 μg protein, must be kinetically controlled and mass-transfer limitations in the system are not yet occurring [13]. This interpretation is consistent with the model presented by Ruckenstein and Karpe [14] in which the inter- and intramicellar diffusion of molecules is much faster than the enzyme reaction per se.

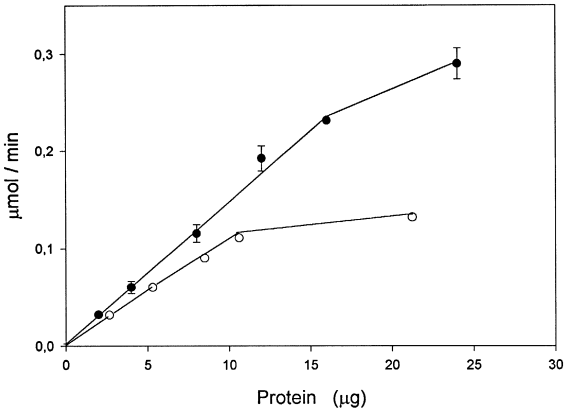

Fig. 1. Dependence of the activity with the amount of protein. The enzymatic assay was carried out in standard conditions except that the amount of protein was varied between 2.6 and 22 μg in aqueous medium (○) and between 2 and 24 μg in reverse micelles (●).

When we compare the enzymatic activity in both media, in spite of the differences between them concerning amount of protein, assay volume and assay time, we note that up to 10 μg the differences in activity are almost negligible. However, above it, activity measured as product units obtained per minute in reverse micelles shows a gain relative to that found in buffer. It seems that this effect is due to an increase in the stability of quinones in cyclohexane relative to water.

3.2. Activity with pH

Fig. 2A shows the pH profiles of polyphenol oxidase activity towards 4-methylcatechol in aqueous and reversed micellar media. The reaction rate is expressed as percent of that shown by control samples at pH 6.5. We note that both profiles are quite alike and almost superimposed. If the free acid of the surfactant were really relevant in changing the real pH inside the micelles, the pH profile would be seen displaced towards alkaline values [15]. On the contrary, we see in Fig. 2A a very light shift towards acid pHs, and this allows us to think that 25 mM phosphate employed to form the micelles is enough to buffer the acid impurities of AOT. Moreover, the substrate is present, and substrates are known to be stabilizers of the enzymes.

Relative to data available in literature dealing with the behaviour of polyphenol oxidase in reverse micelles, the profile of the catechol oxidation in Brij 96/cyclohexane reverse micelles towards pH variation [6] is quite similar to ours in spite of the non-ionic nature of the surfactant. However, in AOT/isooctane reverse micelles, the pH profile of polyphenol oxidase was shifted towards alkaline values and this was attributed to the electric field created by the surfactant and the subsequent rising in the pKs of groups in the enzyme [7]. We must point to the low molarity of the buffer Bru et al. [7]

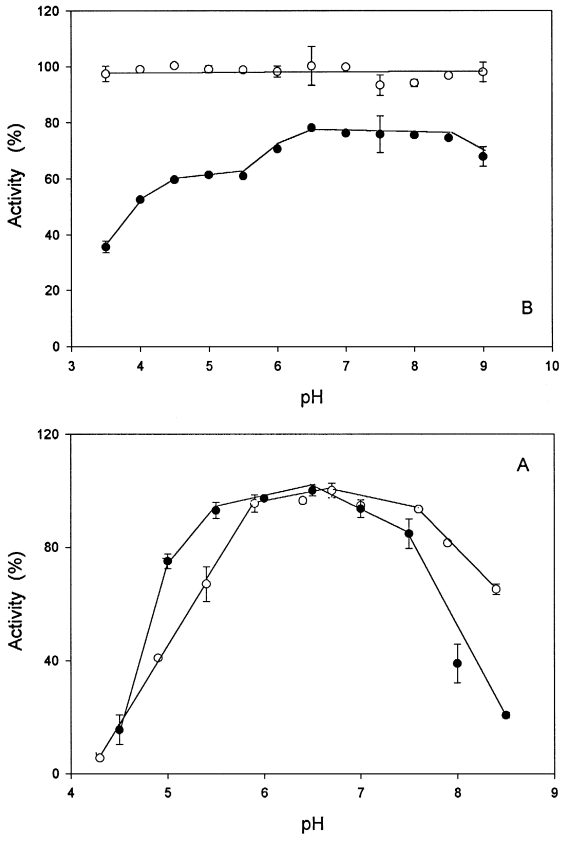

Fig. 2. Activity and stability with pH. Activity with pH (A) in aqueous medium (○) was carried out with 10.8 μg protein in the enzymatic assay in standard conditions except that either 25 mM citrate (pH 4.2 to 6.0), phosphate (pH 6.0 to 8.0) or HCl–Tris (pH 8.0 to 8.4) buffers were employed. The assay in reverse micelles (●) was carried out with 10.8 μg protein in standard conditions except that W_o was 15 and 25 mM of either citrate (pH 4.5 to 6.0), phosphate (pH 6.0 to 8.0) or HCl–Tris (pH 8.0 to 8.5) buffers were employed. Stability to pH (B) in aqueous medium (○) was carried out by preincubating 12 μg protein in 0.5 ml of 10 mM either sodium citrate buffer (pH 3.5 to 6.0), sodium phosphate buffer (pH 6.0 to 8.0) or HCl–Tris buffer (pH 8.0 to 9.0) for 30 min at 17°C. The enzymatic assay begins with the addition of 0.5 ml of 2 mM 4-methylcatechol in 100 mM sodium phosphate buffer pH 6.5 (the ratio between the incubation and preincubation buffer molarities is 10:1) and subsequent incubation at 30°C for 5 min. Stability in reverse micelles (●) was carried out in the following way: To 2.5 ml of 50 mM AOT in cyclohexane were added 41 μl of 10 mM citrate, phosphate or HCl–Tris buffer (same pHs as above) followed by gentle stirring to form the micelles. Then, 4 μL of the same buffer containing 12 μg of enzyme were added and the resulting micelles ($W_o = 20.3$) were preincubated for 30 min at 17°C. The addition of 4 mM 4-methylcatechol in 2.5 ml of 50 mM AOT/cyclohexane containing 45 μl of 100 mM phosphate buffer pH 6.5 (same buffer molarity ratio as above) begins the enzymatic assay that was carried out at 45°C during 10 min with a final $W_o = 20.6$.

employed (10 mM) instead of the 25 mM that we used. Moreover, they employed an AOT previously purified but instead of freeze-drying it, as we did, they dessicated it, and this slow process may have occasioned some ionization not properly buffered.

3.3. Stability to pH

Various stock solutions of different buffers and pHs ranging from 3.5 to 9 were employed to check the stability with pH of polyphenol oxidase in aqueous medium and reverse micelles. Results are depicted in Fig. 2B and are expressed as percent activity relative to control samples not preincubated and assayed at pH 6.5.

The enzyme is stable upon preincubation for 30 min in the pH range 3.5–9.0 in aqueous medium and if preincubation takes place for 3 h, the flat profile is also kept but the residual activity is only 85% with regard to control samples (not shown), which indicates that the enzyme is very stable to pH changes but not so much to the preincubation time.

Stability of polyphenol oxidase to pH in reverse micelles was checked by preincubating the enzyme for 30 min in 2.5 ml of 50 mM AOT/cyclohexane reverse micelles formed with 10 mM of the corresponding buffer and pH (see legend of Fig. 2B). The enzymatic assay was carried then by the addition of 2.5 ml of reverse micelles formed with 100 mM sodium phosphate buffer, pH 6.5 containing the substrate. The final pH in the assay should be 6.5 since the buffer molarity ratio in the preincubation and incubation is 1/10.

Nevertheless, which is the real pH inside the micelles is a controversial conceptual point since in principle, there is no direct way either to define or to determine the pH in the water pool (pH_{wp}), mainly because the water in the water pool is a novel solvent, for which no calibration has yet been offered. It is accepted that the pH_{wp} may differ from the pH of the stock solution (pH_{st}) due to acidic AOT degradations products perturbing the pH in the water droplets and that the AOT purification must overcome this effect [16]. If purification did not take place, it is considered that the effects of impurities of the surfactant can be negated provided that the buffer has enough buffering capacity, e.g., 50 mM and that it was used at its pK [17].

Given the high molarity of the buffer we employed in the incubation and the low one in the preincubation, we must assume that the fall in residual activity at acidic pHs in Fig. 2B is due to the decrease of the real acid pH in the preincubation . A possibility to assess it and to overcome this inactivation could be to increase the preincubation buffer molarity up to 25; however, as we have the methodological need of achieving pH 6.5 in the ulterior enzymatic assay, we should employ a 250-mM buffer to maintain the 1/10 buffer molarity ratio and then the activity would decrease due to the sensitivity of the enzyme to ionic strength [3]. Moreover, we must consider that the electric environment created by AOT may contribute to elevation of the pKs of the enzyme [7], which in turn should enhance the denaturing effect of acidic pHs.

In Fig. 2B we note also a loss of 20% in residual activity at neutral pHs. Even if the real pH is lowered by impurities of AOT, the real pH may not be too low to cause the enzyme inactivation. We think that the activity loss must be a consequence of the lack of stability of the protein in reverse micelles in absence of the substrate, due to its interaction with the solvent, the surfactant or both and which is minimized when the substrate is present. Several temperature studies we actually carried out, which are the content of an article in preparation, point to this explanation.

Relative to the stability with pH that other enzymes show in reverse micelles, we found no data in literature to be compared with our results.

3.4. Influence of water and [AOT] on the activity

The influence of water on enzymatic reaction in reverse micelles is characterized by the molar ratio of water to surfactant (i.e., W_o). In the present study, the reaction rates of polyphenol oxidase in AOT/cyclohexane reverse micelles with 4-methylchatecol as substrate were determined at W_o between 5 and 28. Higher W_o values were not included since microemulsions became turbid in the presence of the substrate. The surfactant concentration was kept constant (50 mM) whereas the water concentration was varied. It is known that increasing the degree of hydration of the micelles leads to an increase in the size of the inner micellar cavity as well as in the charge of the reverse micelle surface [14].

Results depicted in Fig. 3A show increasing reaction rates of polyphenol oxidase as we increase W_o up to 20, followed by a small decrease thereafter. The activity vs. W_o profile in AOT/cyclohexane approaches the bell-shaped profile that shows polyphenol oxidase in AOT/isooctane reverse micelles [7], but for the maximum of activity, which is 20 in our system instead of 35. Other results in Brij 96/cyclohexane point to 10 as the maximum W_o [6]. To account for these differences, we must remember that the maximum W_o for an enzyme in reverse micelles depends on various factors like the size of the enzyme, the nature of the surfactant and the composition of the medium. Moreover, the methodology of the experiment may also be relevant since we kept the AOT concentration constant (50 mM) and varied the water concentration whereas Bru et al. [7] did just the opposite. The different protocol may affect the distribution of the substrate since it is known that aromatic substrates can act as cosurfactants by interacting with the surfactant tails, thus modifying the available substrate to the enzyme as the surfactant concentration varies [14].

The activity of polyphenol oxidase towards 4-methylchatecol was also checked in reverse micelles AOT/cyclohexane as the surfactant concentration varied. Since W_o is to be kept constant ($W_o = 20$), the water concentration was also varied. Results are depicted in Fig. 3B and we can observe that the enzymatic activity does not vary practically with the increasing [AOT]. It is known that the increase in surfactant at constant W_o has no effect on the size of the water cavity of the micelles but raises the micelle concentration increasing the interfacial area. The factors influencing the enzyme behaviour are both the nature of the enzyme itself and of the substrate. With regard to the nature of the protein, those hydrophobic enzymes having association with membranes decrease their activity hyperbolically when the surfactant concentration is increased [18] due to enzyme deterioration after collision between the reverse micelles filled with enzyme and the unfilled ones, which might be accelerated by the increase in the number of the reverse micelles as the [AOT] increases. On the other hand, the typical water-soluble enzymes have a catalytic behaviour independent of

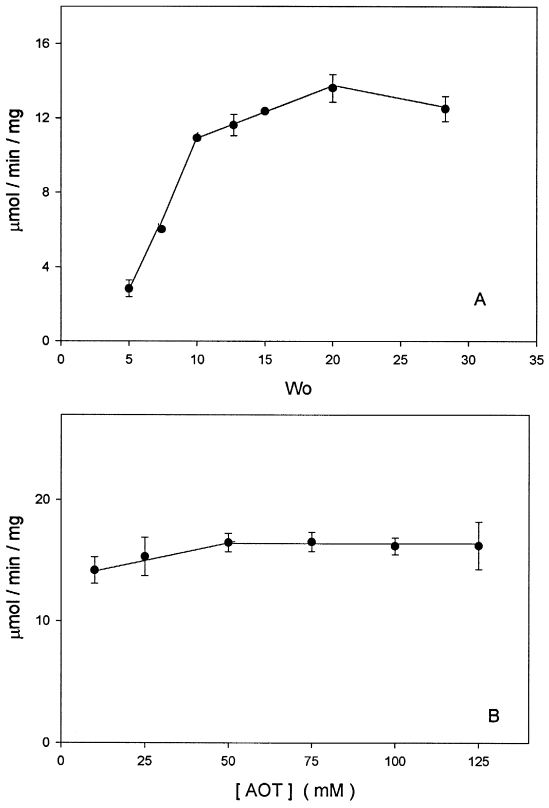

Fig. 3. Effect of W_o and the surfactant concentration on the activity. The effect of W_o on the enzyme activity (A) was checked in 5 ml of 50 mM AOT/cyclohexane containing 2.5 mM 4-methylcatechol and a variable amount of 25 mM phosphate buffer pH 6.5. The protein (8.1 μg) was added in 15 μl of the same buffer and the assay was carried out at 37°C for 10 min. In the enzymatic assay, the molar ratio $[H_2O]/[AOT]$ varied from 5 to 28. The effect of surfactant concentration on the enzyme activity at constant W_o (B) was studied by varying [AOT] from 10 to 125 mM in the enzymatic assay. The amount of 25 mM phosphate buffer pH 6.5 added to form the reverse micelles was also varied to keep W_o constant. The reaction mixture was 2.5 mM in 4-methylcatechol and the assay began with the addition of 8.1 μg protein in 15 μl of the same buffer (final $W_o = 20$). Other conditions as in the standard assay.

surfactant concentration at constant W_o, and polyphenol oxidase appears to form part of this group as deduced from Fig. 3B.

3.5. Kinetic studies

The kinetic behaviour of polyphenol oxidase towards 4-methylcatechol was studied in buffer up to 16 mM substrate and in reverse micelles AOT/cyclohexane up to 5 mM (overall concentration in micellar medium) of 4-methylcatechol. Fig. 4 shows the activity vs. substrate concentration plot. We see that the enzymatic activity increases to the same value (1 mM) in both aqueous (A) and micellar (B) media, indicating that there is neither loss of activity nor superactivity in our reverse micellar medium, whereas it has been previously reported that the activity decreased four times after the inclusion of polyphenol oxidase in AOT/isooctane reverse micelles [7]. In Fig. 4A, above 1 mM, polyphenol

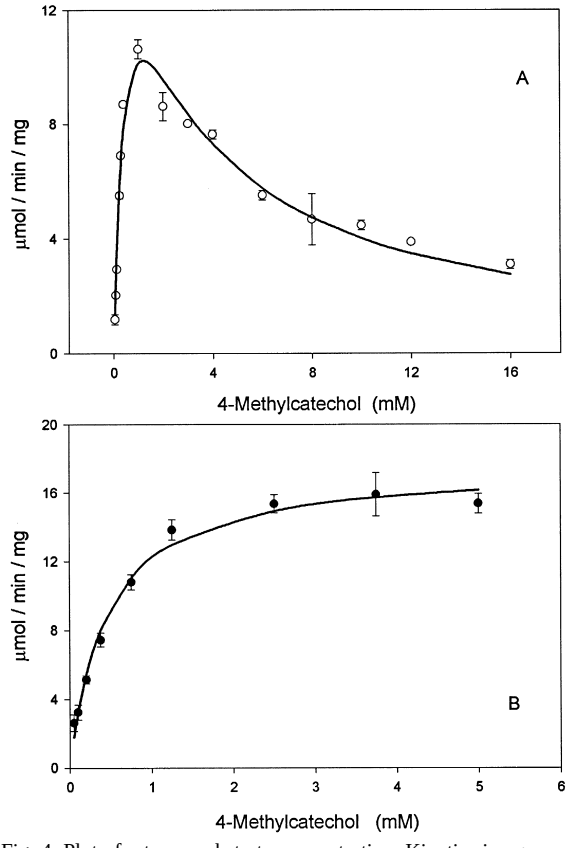

Fig. 4. Plot of rate vs. substrate concentration. Kinetics in aqueous medium (A) towards 0.04–16 mM 4-methylcatechol were assayed with 8.1 μg protein in 1 ml 25 mM phosphate buffer pH 6.5 at 30°C for 5 min. The 4-methylquinones obtained were measured at 400 nm. Kinetics in reverse micelles (B) towards 0.025–5 mM 4-methylcatechol were assayed in 5 ml 50 mM AOT/cyclohexane with 25 mM phosphate buffer pH 6.5 and 8.1 μg protein at 45°C for 10 min ($W_o = 20$). The 4-methylquinones obtained were measured at 378 nm.

oxidase shows substrate inhibition in aqueous medium whereas a classical v vs. s plot is obtained in reverse micelles (Fig. 4B). The lack of substrate inhibition in reverse micelles may be explained by the substrate partition among the water pool, the solvent and the hydrophobic tails of AOT, since substrates containing aromatic rings do associate with AOT [19]. The consequence of the substrate partition and its association with the surfactant may be the decrease of the real substrate concentration in the water pool so that the concentration at which inhibition occurs, is not actually reached inside the reverse micelles. No substrate inhibition was reported for polyphenol oxidase in Brij 96/cyclohexane reverse micelles by Bru et al. [6] whereas the same authors found a light substrate inhibition in AOT/isooctane reverse micelles [7].

The experimental data in Fig. 4 were fitted by nonlineal regression either to the Michaelis Menten equation Michaelis Menten equation (1) in micellar medium (Fig. 4B) or to the equation for substrate inhibition (2) in aqueous medium (Fig. 4A) according to Cleland [20] by using the SIGMA-PLOT 4.0 programme. The curves depicted in Fig. 4 are the fitted ones.

$$v = VS/(K_m + S) \quad (1)$$

$$v = VS/\left(K_m + S + S^2/K_I\right) \quad (2)$$

where v is initial velocity, V is the apparent maximum velocity of the reaction, K_m is the apparent Michaelis constant, S is substrate concentration and K_I is the inhibition constant due to substrate excess.

Table 1
Apparent kinetic parameters of polyphenol oxidase

Substrate	Medium	V (μmol min^{-1} mg^{-1})	K_m (mM)	K_i (mM)	log P
Catechol	Aqueous	34.96 ± 1.02	0.50 ± 0.07		0.96
	Micellar	13.41 ± 0.37	0.18 ± 0.02		
Phenol	Aqueous	1.19 ± 0.03	0.20 ± 0.03		1.49
	Micellar	3.77 ± 0.10	0.28 ± 0.054		
4-Methylcatechol	Aqueous	19.12 ± 2.40	0.52 ± 0.11	2.7 ± 0.57	1.49
	Micellar	17.61 ± 0.53	0.45 ± 0.05		
p-Cresol	Aqueous	3.60 ± 0.82	0.60 ± 0.18	0.5 ± 0.17	2.02
	Micellar	3.11 ± 0.17	0.64 ± 0.14		
t-Buthylcatechol	Aqueous	22.84 ± 0.53	0.45 ± 0.045		3.23
	Micellar	16.96 ± 0.53	5.29 ± 0.37		
L-Tyrosine	Aqueous	4.88 ± 0.11	0.22 ± 0.016		
L-DOPA	Aqueous	9.38 ± 0.33	0.29 ± 0.035		

The enzymatic assays were carried out in 1 ml of 25 mM sodium phosphate buffer pH 6.5 at 30°C in aqueous medium and in 5 ml of 50 mM AOT/cyclohexane with the same buffer ($W_o = 20$) at 45°C in reverse micelles. Other conditions were varied depending on the substrates. (a) With catechol: 9.2 μg protein, from 0.04 to 50 mM substrate, 5 min incubation and recording of the quinones at 388 nm in aqueous medium. In reverse micelles, 8.1 μg protein were incubated with 0.05–2 mM substrate for 10 min and the absorbance was recorded at 372 nm. (b) With 4-methylcatechol: conditions described in the legend of Fig. 4. (c) With phenol: 9 μg protein and 0.1–18 mM substrate were incubated for 30 min and the absorbance was recorded at 388 nm in aqueous medium. In reverse micelles, 32 μg protein were incubated with 0.05–50 mM substrate for 30 min and the absorbance was recorded at 372 nm. (d) With *p*-cresol: 9 μg protein were incubated with 0.05–16 mM substrate for 30 min and the absorbance was recorded at 400 nm in aqueous medium. In reverse micelles, 8.1 μg protein were incubated with 0.35–7 mM substrate for 10 min and the absorbance was measured at 378 nm. (e) With 4-*t*-butylcatechol: 10.6 μg protein were incubated with 0.02–18 mM substrate for 5 min and the absorbance was measured at 400 nm in aqueous medium. In reverse micelles, 10.6 μg protein were incubated with 0.12–16 mM substrate for 10 min and the absorbance was measured at 366 nm. (f) With L-tyrosine: 26.6 μg protein were incubated with 0.02–1.8 mM substrate for 5 min and the absorbance was recorded at 478 nm in aqueous medium. (g) With L-DOPA: 10.6 μg protein were incubated with 0.01–1.8 mM for 5 min and the absorbance was measured at 478 nm in aqueous medium.

The log P of substrates was calculated fromthe structural group contributions according to Schwarzenbach et al. [22]. The difference with the experimental ones, when available in literature, is very small (experimental log P of phenol is 1.45 and experimental log P of *m*-cresol is 1.96) [22].

The kinetic study was also carried out with other substrates: *p*-cresol, phenol, catechol and 4-*t*-butylcatechol in aqueous medium and in reverse micelles whereas L-tyrosine and L-DOPA could only be checked as substrates in buffer given their low solubility in cyclohexane and 4-*t*-butylphenol could not be checked neither in water nor in reverse micelles. The fitting of the data to the corresponding equations gave the apparent kinetic parameters summarized in Table 1. We found substrate inhibition only with *p*-cresol in buffer but not in reverse micelles, same as with 4-methylcatechol. Substrate inhibition may result from the dead-end combination of the substrate with an enzyme form with which it is not supposed to react, or from the binding of two substrate molecules to a pocket of the enzyme designed for the binding of only one [20]. No matter what the cause of inhibition is, it is plain that in aqueous media the addition in *p* position of a methyl group to phenol or catechol is the responsible of substrate inhibition in buffer.

We see in Table 1 that the apparent K_m increases in reverse micelles as the hydrophobicity of the substrate (a measure of which is the log of partition coefficient) also increases. We presume that the different partition of the substrate among water, interface and solvent might concentrate it either preferentially in the water pool (catechol) or in the solvent or interface (*t*-butylcatechol). As we consider the overall substrate concentration in micellar medium, it would be necessary to have a lower overall catechol concentration or a higher *t*-butylcatechol concentration than in buffer to achieve the half-saturation concentration inside the micelles with both substrates. When we compare the apparent K_ms in micelles with those obtained in buffer, we see noteworthy differences regarding catechol (2.7 times lower) and 4-*t*-butylcatechol (15 times higher), whereas with substrates having a calculated log *P* above 1 and below 3 the apparent K_ms are practically maintained in reverse micelles relative to those found in water.

Fig. 5 represents the dependence of the apparent catalytic efficiency of polyphenol oxidase (V/K_m) with the hydrophobicity of the substrate. We see that there is a strong dependence of the log *P*, which seems logical given the dependence the apparent K_m also showed. Thus, the best substrate for polyphenol oxidase in reverse micelles of AOT/cyclohexane is catechol followed by 4-methylcatechol, phenol, *p*-cresol and *t*-butylcatechol. Catechol is also the best substrate in buffer as deduced from data in Table 1 and this point confirms data previously reported in literature [21].

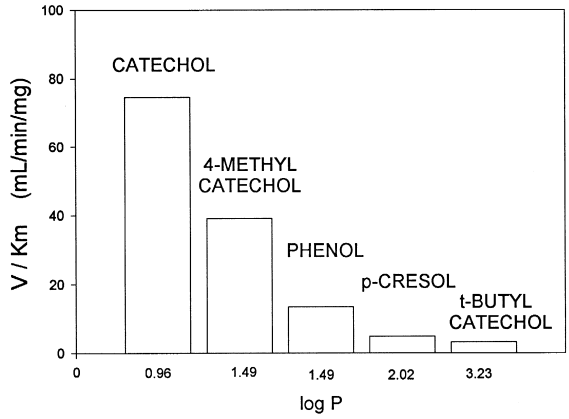

Fig. 5. Dependence of the apparent catalytic efficiency of polyphenol oxidase in reverse micelles on the substrate hydrophobicity . The apparent catalytic efficiency (V/K_m) of the enzyme in reverse micelles was calculated from the apparent kinetic parameters for the substrates summarized in Table 1. The log *P* of the substrates was calculated as described in the legend of Table 1.

4. Conclusions

Polyphenol oxidase is able to catalyze several phenols and catechols in AOT/cyclohexane reverse micelles, and with 4-methylcatechol as substrate shows neither superactivity nor inhibition with regard to its behaviour in buffer. The enzyme shows inactivation when it is preincubated in reverse micelles in the absence of the substrate regardless of the preincubation pH and this inactivation is more marked at acidic pHs. The cause of the last may be the acid impurities of surfactant and the electric environment of the enzyme due to AOT whereas the cause of the inhibition at neutral pHs may be due to the confinement process of the enzyme in the micelles. The pH activity profile in reverse micelles is practically maintained with regard to the obtained in water. The optimum W_o is 20 and the enzymatic activity does not depend on the micellar concentra-

tion, which corroborates the hydrophilic character of the protein. The fact that the apparent catalytic efficiency of the enzyme is strongly and directly dependent of the substrate hydrophility, whereas the apparent K_m is inversely dependent of it, suggests the relevance of the substrate partition among the solvent, the inner core and the surfactant tails.

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