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Effect of ionic and non-ionic surfactants on the activity and stability of mushroom tyrosinase

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Abstract

Three typical surfactants were selected based on their head group charges: an anionic surfactant sodium di-2-ethylhexylsulfosuccinate (AOT), a non-ionic surfactant polyethylene glycol hexadecyl ether (Brij 52), and a cationic surfactant cetyltrimethylammonium bromide (CTAB). The kinetic parameters (such as K_m, V_{max}, optimal pH and temperature, and activation energy) and the thermostability of the enzyme at different temperatures (including half-lives, deactivation constants, and activation energies for enzyme deactivation) were determined and compared in the absence and presence of the three surfactants. Both AOT and Brij 52 showed the ability of activating the enzyme while CTAB inactivated it. In terms of stability, Brij 52-induced stabilization and AOT and CTAB-mediated destabilization of the enzyme were observed. Thermoinactivation for both the native and surfactant-treated tyrosinases generally obeyed the classical first-order kinetics. The effects of the three surfactants may be related to a conformational change on the enzyme.

Keywords: Tyrosinase; Polyphenol oxidase; Ionic surfactant; Non-ionic surfactant

1. Introduction

Tyrosinase, also called polyphenol oxidase (EC 1.14.18.1), is a copper-containing enzyme widely distributed in plants, animals and microorganisms [1]. It possesses two activities: cresolase activity that catalyzes *ortho*-hydroxylation of monophenols, and catecholase activity that mediates oxidation of *o*-diphenols to *o*-quinones. Tyrosinase is the enzyme that catalyzes the production of L-dopa, a medication for the Parkinson's disease [1], and is of great interest in organic synthesis because of its specific regioselectivity [2]. Previous studies have demonstrated that tyrosinase extracted from mushrooms is active not only in aqueous solution but also in organic media such as in an organic solvent containing a low water content [2,3] and in reversed micelles formed by surfactants in an apolar solvent [4].

Our previous studies [3,4] have demonstrated that mushroom tyrosinase functions in two types of reversed micelles: those formed by the anionic surfactant sodium di-2-ethylhexylsulfosuccinate (Aerosol OT, AOT) in isooctane and those formed by the cationic surfactant cetyltrimethylammonium bromide (CTAB) in hexane with chloroform as the co-surfactant. As compared to the activity obtained in aqueous solution, the enzyme showed higher and lower activities in the AOT/isooctane and CTAB/hexane/chloroform reversed micelles, respectively. Sánchez-Ferrer et al. [5] have also reported that grape tyrosinase was active in reversed micelles composed of the non-ionic surfactant Brij 96 in cyclohexane. Therefore, it is the interest of our current study to investigate whether the variation of enzyme activity in different reversed micelles, as compared to that in aqueous solution, result from the effect of these three different types of surfactants.

Enzyme properties in aqueous surfactant solutions have been studied by some authors in order to provide information about the role of surfactants on enzyme activity in reversed micelles. While AOT showed the same inhibiting effect on alcohol dehydrogenase [6] and α-chymotrypsin [7] in both normal and reversed micelles, other enzymes such as lipase [7], catalase and horseradish peroxidase [8] performed differently in the two systems. Therefore, no obvious correlations have been available yet between enzyme activity in aqueous surfactant solutions and in reversed micelles, and the factors responsible for the enzyme

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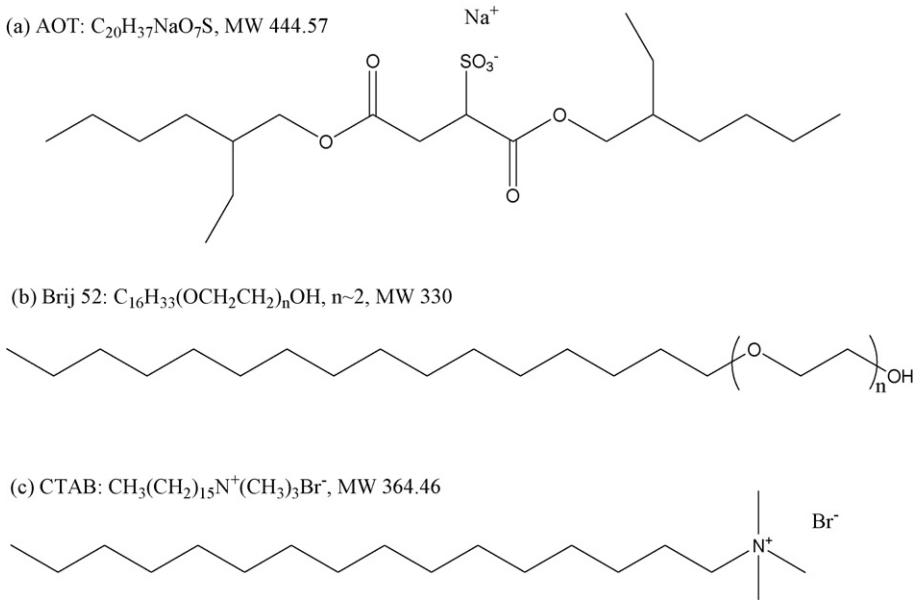

Scheme 1. Molecular formula and structures of the three surfactants used in this study.

properties in the two systems seem to depend on both the enzyme and the surfactant.

With regard to tyrosinase, it has long been reported that sodium dodecyl sulphate (SDS), an anionic detergent, is an activating agent to the enzyme [9], and recent experiments have confirmed that the activation is accompanied by an interaction between the detergent and the enzyme, resulting in a conformational change in the enzyme [10–12]. Investigations related to tyrosinase from mushrooms have revealed that this enzyme can also be activated by the anionic surfactants SDS [13] and AOT [14] but inactivated by the cationic surfactant cetylpyridinium chloride (CPC) [15]. These findings seem to suggest that the activity of mushroom tyrosinase is facilitated by anionic surfactants and not by cationic ones.

Although surfactant–enzyme interactions in aqueous solutions have been extensively studied [16], a detailed comparison of enzyme activity and stability in the presence of different types of surfactants is still lacking. Therefore, the aim of this study is to examine in detail the effect of three different types of surfactants (anionic AOT, non-ionic Brij 52, and cationic CTAB) on the activity and stability of mushroom tyrosinase in aqueous solution. The molecular formula of the three surfactants are shown in Scheme 1. The results obtained from this study are expected to provide information on the enzyme–surfactant interaction and on the enzymatic performance in reversed micelles.

2. Experimental

2.1. Materials

Fresh mushrooms (*Agaricus bisporus*) were purchased from a local supermarket in Shenzhen, China. L-dopa (3,4-dihydroxy-L-phenylalanine) and three surfactants (AOT, CTAB, and Brij 52) were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Enzyme preparation

The enzyme was obtained as the supernatant after centrifugation of an extract of fresh mushrooms (50 g) in 100 ml phosphate buffer (50 mM, pH 6.0) [4]. The enzyme preparation used in this study was in fact a tyrosinase-rich protein complex without being further purified.

2.3. Enzyme activity assay

The activity of the enzyme solution was determined by following the formation of dopachrome spectrophotometrically at 30 °C (3). After addition of 50 μl enzyme extract to a cuvette containing 1.2 ml phosphate buffer (50 mM, pH 6.0) and 0.8 ml 10 mM L-dopa (prepared in the same phosphate buffer), the solution was immediately mixed and the increase in absorbance at 475 nm (indicating the formation of dopachrome) was recorded with the Pharmacia Biotech Ultraspec 2000 UV/Vis spectrophotometer equipped with a thermostated cell. The initial rate was calculated from the linear part of the progress curve (i.e. over the first 2–3 min). One unit of tyrosinase was defined as the amount of the enzyme catalyzing the oxidation of 1 μmol L-dopa to dopachrome per minute at 30 °C. The extinction coefficient of dopachrome at 475 nm was 3600 M^{-1} cm^{-1} [17]. In the following experiments for studying the effect of surfactants on the kinetic parameters and optimum pH and temperature of the enzyme, both phosphate buffer and the substrate solution were prepared containing a fixed concentration of a surfactant: 1.5 mM for AOT, 20 mM for CTAB, and 100% saturation for Brij 52. Due to the limited solubility of Brij 52 in water, its aqueous solution (final concentration much lower than 1.2 mM) was prepared by sonicating 0.1 g of Brij 52 in 250 ml phosphate buffer at room temperature for 2 h and then filtering through a filter paper. Experiments have confirmed that the presence of either the substrate or the surfactants did not affect the pH of the

phosphate buffer significantly. All the experiments throughout this study were repeated at least twice with experimental errors ≤5%.

2.4. Determination of K_m, V_{max}

The reaction was carried out at 30 °C in a cuvette where 50 μl of enzyme solution was added to 2 ml 50 mM phosphate buffer containing various concentrations of the substrate L-dopa (0–4 mM). The K_m and V_{max} values of the enzyme were obtained from the Lineweaver–Burk plots.

2.5. Optimum pH and temperature

Optimum pH was determined by measuring tyrosinase activity at 30 °C over a pH range of 5.0–8.0 in 50 mM phosphate buffer. Optimum temperature was obtained by measuring the activity of tyrosinase in 50 mM phosphate buffer (pH 6.0) at various reaction temperatures (5–70 °C).

2.6. Stability

2 ml of the enzyme solution (prepared in 50 mM phosphate buffer, pH 6.0) was mixed with 4 ml of each of the following solutions: the phosphate buffer only (as a control), and the phosphate buffer containing 1.5 mM AOT, 20 mM CTAB or 100% saturation of Brij 52, respectively. The mixtures were then incubated in a water bath controlled at different temperatures (40, 50, 60 °C), and periodically 100 μl of each solution was taken for activity assay at 30 °C as noted above. The time-dependent loss in activity was used to calculate the half-life for each enzyme solution. According to the classical first-order kinetics of enzyme deactivation, the first-order deactivation rate constants were determined as the slopes of the ln V versus t plots, where V is the residual enzyme activity after incubation at a specific temperature for a specific period and t is the incubation time. Activation energies for the thermal deactivation were measured by plotting logarithms of the deactivation rate constants versus reciprocals of the absolute temperatures according to the Arrhenius equation.

3. Results

3.1. Effect of surfactants on product formation

The activity of tyrosinase was represented by the formation of the product dopachrome, which showed a strong absorbance at 475 nm in aqueous solution. Our experiments have confirmed that the absorption peak for dopachrome at 475 nm was not shifted by addition of any of the three surfactants (AOT, CTAB, and Brij 52). Therefore, it is feasible to determine the activity of tyrosinase by following the increase in absorbance at 475 nm of the reaction system with and without the involvement of the surfactants.

3.2. Effect of surfactants on enzyme activity

Variation of the enzyme activity upon the change in the concentration of each surfactant was investigated first. Interestingly, different surfactants affected the activity of tyrosinase in different ways. The enzyme activity rose gradually as the concentration of AOT was increased, reaching its maximum when [AOT] = 1.0 mM and then leveling off until [AOT] = 1.5 mM, which was the maximum AOT concentration used in the buffer tested due to the limited solubility of AOT. In the case of Brij 52, altering the surfactant concentration did not show a significant effect. This may be due to the extremely low concentration of Brij 52 in the buffer even when the surfactant was at 100% saturation. Addition of CTAB to the reaction system, however, resulted in a gradual reduction in enzyme activity. These results have therefore suggested that the anionic AOT and cationic CTAB might have the potential of activating and inhibiting the enzyme, respectively, whereas the non-ionic Brij 52 did not affect the enzyme activity significantly.

In order to further confirm the above effects of surfactants on enzyme activity, the K_m and V_{max} values of the enzyme in the presence and absence of the 3 surfactants have been determined and compared (Table 1). Purposely, our experiments were designed to use phosphate buffers containing 1.5 mM AOT, 20 mM CTAB, and 100% saturation of Brij 52, respectively. These were the maximum surfactant concentrations used in the above experiments.

As compared to the control group in which no surfactants were added, tyrosinase did present a higher and a lower ratio of V_{max}/K_m in the presence of AOT and CTAB, respectively, thus sustaining that AOT may work as an activator to the enzyme while CTAB as an inhibitor. In fact, the activation effect of AOT was reflected by an obvious enhancement in V_{max}. CTAB, on the other hand, inhibits the enzyme by increasing the K_m while keeping the V_{max} fairly constant, and therefore seemed to act as a competitive inhibitor to the enzyme. Interestingly, addition of Brij 52 also resulted in an elevated V_{max}/K_m, which seemed to be more related to a decrease in K_m and less to the change in V_{max}.

Table 1
Comparison of the kinetic parameters of mushroom tyrosinase in the absence and presence of the 3 surfactants

Surfactant	Control	AOT	Brij 52	CTAB
K_m (mM)	0.67 ± 0.03	0.71 ± 0.03	0.50 ± 0.02	1.00 ± 0.05
V_{max} (mM/min)	0.22 ± 0.01	0.29 ± 0.01	0.21 ± 0.01	0.24 ± 0.01
V_{max}/K_m	0.33	0.41	0.42	0.24
Optimum temperature (°C)	50	40	50	40
Activation energy (kJ/mol)	31.8	34.4	24.2	35.9

3.3. Effect of surfactants on pH and temperature optima

The presence of the surfactants has also affected the pH and temperature optima of the enzyme. Regarding the pH dependence of the enzyme, three effects resulting from addition of the surfactants can be observed from Fig. 1: (1) the optimum pH of the enzyme was slightly shifted; (2) the enzyme seemed to become more sensitive to the change in pH, especially so with the addition of AOT; (3) the relative activities of the enzyme presented at both humps (pH 5.75 and pH 7.31) seemed to be reversed to different degrees by the addition of the surfactants, more seriously with AOT than with CTAB. A shift in pH optimum for an enzyme upon addition of surfactants has not been uncommon, as Jiménez and García-Carmona [18] and Espín and Wichers [13] have reported a significant right shift in the optimum pH of tyrosinase induced by the addition of SDS. Kanade et al. [12] have suggested that field bean tyrosinase exists in two forms with two specific pH optima. In agreement with their findings, our results seemed to suggest that mushroom tyrosinase might also exist in two forms, one with an optimum pH of 5.7–6.0 and another one at pH 7.3, and that addition of ionic surfactants such as AOT and CTAB would make the first form more favorable. It is unclear yet what triggered this pH shift, but part of the reason might be related to some pH-dependent molecular interactions between the surfactant and the substrate.

The optimum temperature of the enzyme, with and without addition of the surfactants, can be obtained from Fig. 2, which shows variation of enzyme activity upon the increase in reaction temperature. The data in Fig. 2 can also be used to calculate the activation energy of the reaction catalyzed by tyrosinase in the absence or presence of surfactants, and the results are listed in Table 1. The enzyme retained the same optimum reaction temperature (50 °C) when treated with Brij 52, but both AOT and CTAB shifted the optimum temperature of the enzyme to 10 °C lower. The activation energy of the reaction catalyzed by tyrosinase was increased by the addition of AOT and CTAB and decreased in the presence of Brij 52. Furthermore, when exposed to high reaction temperatures the enzyme seemed to retain its activity in the trend of control > Brij 52 > AOT > CTAB. For instance, at 70 °C the percentage of the retained activity as compared to the activity presented by the enzyme at its optimum reaction temperature was 76.9, 43.7, 20.7 and 6.0% for the enzyme only, and the enzyme with the addition of Brij 52, AOT, and CTAB, respectively.

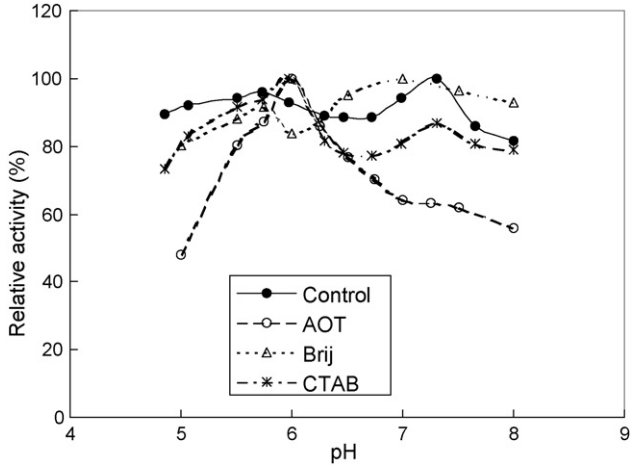

Fig. 1. Dependence of enzyme activity on pH.

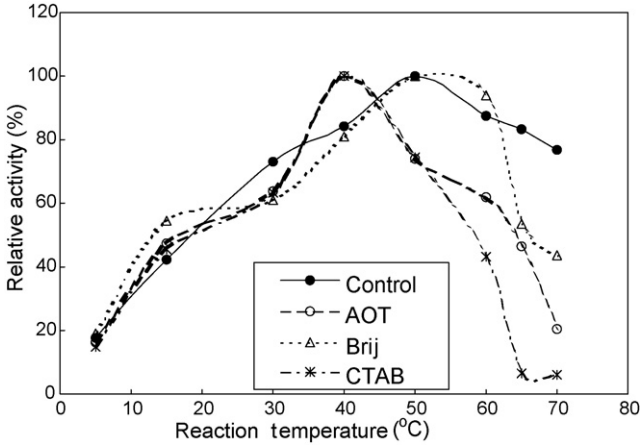

Fig. 2. Dependence of enzyme activity on reaction temperature.

3.4. Effect of surfactants on enzyme stability

The thermostabilities of both the native and surfactant-treated enzymes were studied by measuring the residual activities of the enzymes under fixed conditions after incubation for appropriate periods at different temperatures (40, 50, 60 °C). As expected, all four forms of the enzyme showed a decreased stability when the incubation temperature was increased (Fig. 3).

However, different surfactants may affect the enzyme stability differently. From the plots in Fig. 3 one can observe that the stability of the enzyme was obviously decreased in the presence of AOT (1.0 mM in the incubation buffer), and that the situation was even worse when CTAB (13.3 mM) was involved. On the other hand, Brij 52 (at 66.7% saturation) seemed to show a stabilization effect on the enzyme, and this effect appeared to become more obvious when the incubation temperature was higher. These results are confirmed by plotting the logarithms of the half-lives of the enzyme versus the incubation temperatures (Fig. 4). It is evident that tyrosinase was most unstable when treated with CTAB, less unstable with AOT, and most stable with Brij 52. The half-life of the Brij 52-treated tyrosinase was the same or slightly higher than that of the native enzyme, and especially so when the enzyme was incubated at a higher temperature.

Although there are quite a few schemes trying to describe enzyme deactivation kinetics, the time course of the thermoinactivation for both the native and surfactant-treated tyrosinases generally obeyed the classical first-order kinetics, and typical examples are presented in Fig. 5a. The first-order deactivation rate constants, obtained by fitting our experimental data to the model, are listed in Table 2. The activation energies for thermal inactivation of the four types of tyrosinase were obtained from the Arrhenius plots of logarithms of the deactivation rate

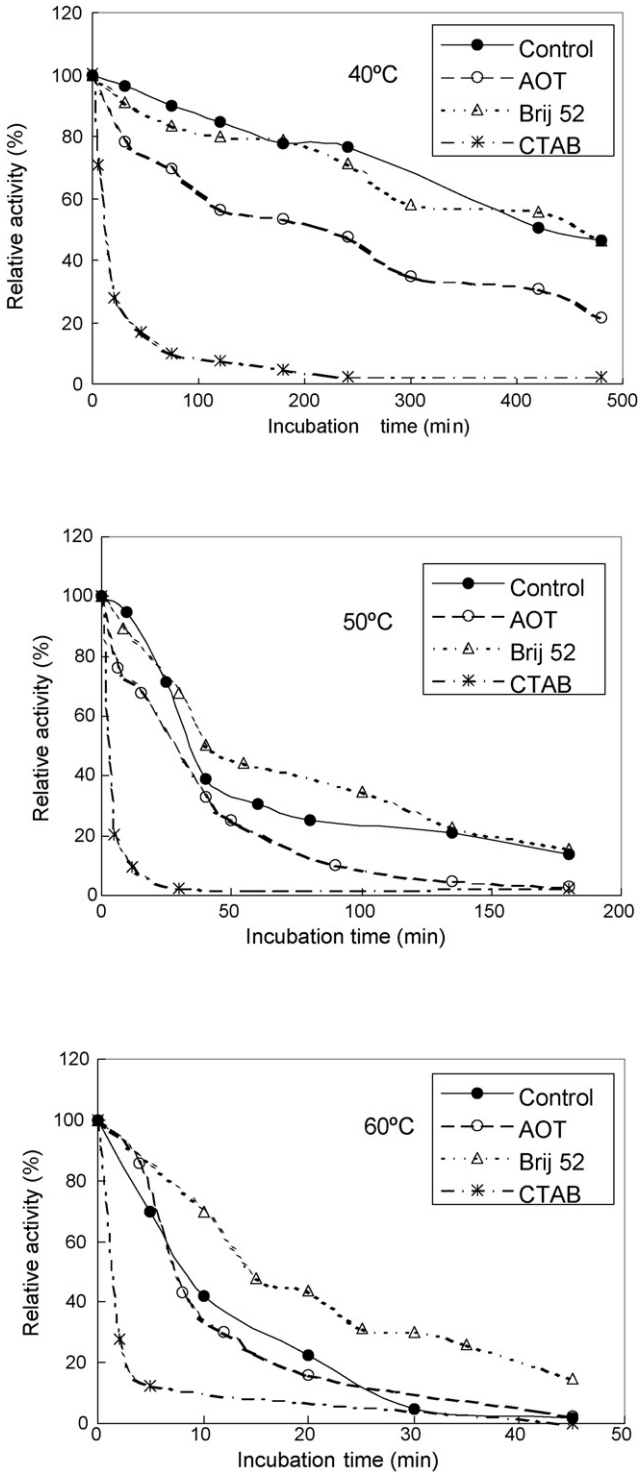

Fig. 3. Effect of surfactants on tyrosinase deactivation at different temperatures.

constants versus reciprocals of the absolute temperatures (see Fig. 5b), and are also listed in Table 2.

4. Discussion

Previous studies on enzyme activity and stability in aqueous surfactant solutions (for a review, see [16]) have revealed that the surfactant may affect the catalytic properties of the enzyme

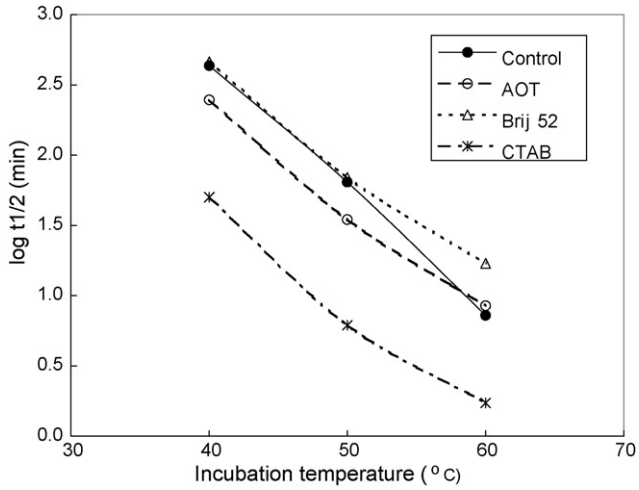

Fig. 4. Effect of incubation temperature on the half-lives of native and surfactant-treated tyrosinases in pH 6.0 phosphate buffer.

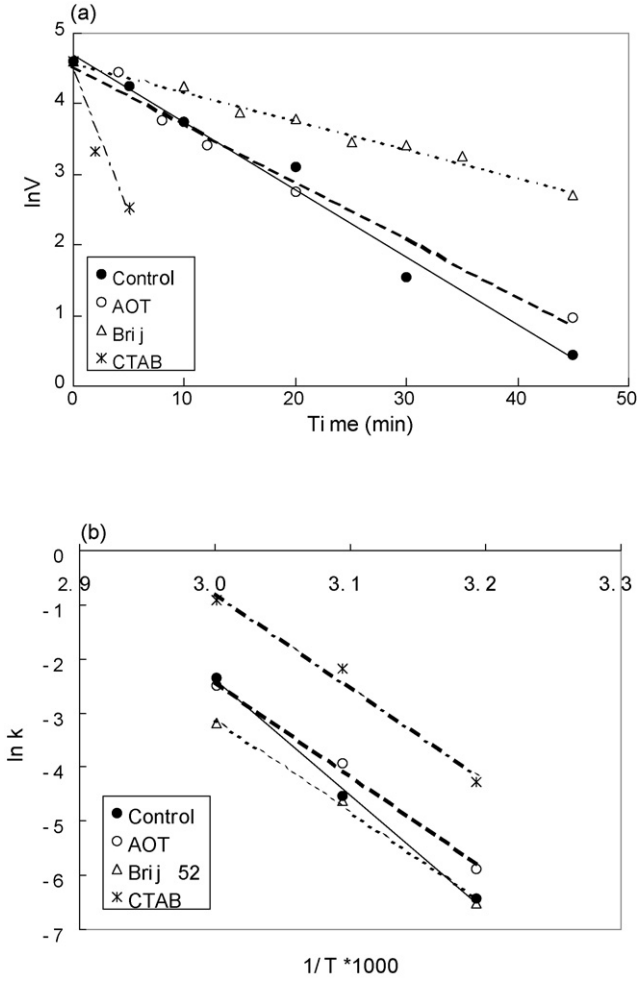

Fig. 5. Kinetics of thermal inactivation of mushroom tyrosinase. (a) Time-dependent loss in enzyme activity at 60 °C. (b) Arrhenius plots of tyrosinase inactivation.

Table 2
Kinetic parameters of thermal inactivation for mushroom tyrosinase

Surfactant	Control	AOT	Brij 52	CTAB
First-order deactivation rate constant ($k \times 10^2$, min^{-1})				
40 °C	0.16	0.28	0.15	1.4
50 °C	1.1	2.0	1.0	11.2
60 °C	9.6	8.2	4.1	40.2
Activation energy (kJ/mol)	177.2	146.4	143.5	146.4

via selective interactions with the enzyme [19], substrate [20] and product [21], and by changing the environmental conditions such as pH and ionic strength. The interactions between enzyme and surfactant involve both the electrostatic interactions between the surfactant head group and the charged amino acid residues of the enzyme and the hydrophobic interactions between the alkyl chains of the surfactant and the hydrophobic amino acid residues of the enzyme, and hence the determinants of the enzyme–surfactant interactions include the structure and charge of the enzyme and the chemistry of the surfactant such as the charge and size of its head group and the length and hydrophobicity of its alkyl chain. Such interactions may induce a change in the conformation and/or active site of the enzyme [11], thereby affecting the enzyme's activity and stability. Therefore, the effect of surfactants on enzyme properties is specific for each enzyme/surfactant combination with no rationale available up to date.

In our study, three typical surfactants were selected based on the charges on their head groups, and their effects on the activity and stability of mushroom tyrosinase in aqueous solution were examined. According to the reported critical micellar concentration (CMC) data for both AOT (0.64 mM) [22] and CTAB (1 mM) [23] in aqueous solution, both these surfactants in our surfactant-containing buffer solutions were expected to exist in the form of normal micelles. As the real concentration of saturated Brij 52 in the buffer (much lower than 1.2 mM) was unknown, it was uncertain whether or not this surfactant was present as micelles in the buffer. However, this would not affect our discussion related to its effects. It is worth stressing that in this study, the effect of the three surfactants was compared at their concentration levels differing at least one order of magnitude, and therefore the conclusions drawn below may have to be limited to the studied range of the surfactant concentrations.

A comparison of the data for K_m and V_{max} has shown that both AOT and Brij 52 had the ability of raising the catalytic efficiency of the enzyme while CTAB inactivated it. The results induced by AOT and CTAB in aqueous solution were corresponding to the behavior of tyrosinase in AOT and CTAB-formed reversed micelles, in which the enzyme presented a higher ("superactivity") and lower activity as compared to that in aqueous solution, respectively [3]. In this study, CTAB lowered the catalytic efficiency of tyrosinase basically by enhancing its K_m rather than by reducing its V_{max}. The high K_m of tyrosinase in the CTAB solution may result from inhibition by the counterion of the surfactant, bromide. Halide ions are known to be inhibitory to tyrosinase [24]. More importantly, the charged head group of the surfactant plays a determining role in affecting the enzyme–surfactant interactions. As the isoelectric point of mushroom tyrosinase is 4.8 [1], the overall charge of the enzyme is negative at pH 6.0. Due to its positive charge, the cationic surfactant may bind to the enzyme, leading to a change in the conformation and/or active site of the enzyme and in turn an enhancement in K_m. Chen et al. [15] have conducted a kinetic and structural study to demonstrate that mushroom tyrosinase underwent a conformational change while being inactivated by a cationic surfactant cetylpyridinium chloride (CPC). A conformational change in α-chymotrypsin induced by a cationic surfactant tetrabutylammonium bromide (TBABr) has also been confirmed by spectroscopic experiments (CD and fluorescence) [25]. Furthermore, due to such electrostatic interactions, each enzyme molecule may be surrounded by a layer of the surfactant CTAB and its micelles, making the diffusion of substrate molecules into the enzyme's active site more difficult.

Although an increase in the catalytic efficiency of the enzyme was observed with the presence of either AOT or Brij 52, the effects of the two surfactants were different. The Brij 52-induced activation appeared to be more related to a decrease in the enzyme's K_m value. This could be attributed to an increase in the affinity between enzyme and substrate as a result of a conformational change that favored the enzyme catalysis.

Activation effect of another anionic detergent, SDS, on mushroom tyrosinase has been reported by several authors [11,13], and two previous studies [11,12] have conducted circular dichroism and intrinsic fluorescence spectroscopy studies to confirm the partial unfolding of the structure of mushroom tyrosinase upon activation by SDS. Due to this unfolding, Trp and Phe residues are opened up and tyrosine residues are shrunk or placed in a more asymmetric environment [11]. It is reasonable to expect that this conformational change may also happen in our case to the same enzyme when the anionic AOT was present. Therefore, the possibility of a conformational change occurred to the enzyme with the addition of AOT or Brij 52 cannot be neglected. Although both the enzyme and AOT hold negative charges at the pH tested, there are still opportunities for them to interact with each other, thereby altering the enzyme's conformation and active site structure and hence affecting its catalytic properties. For instance, there are hydrophobic interactions between the two alkyl chains of AOT and the hydrophobic amino acid residues of the enzyme such as Tyr, Phe and Trp residues, and electrostatic interactions between the negatively charged AOT and some positively charged amino acid residues of the enzyme such as Lys, Arg, and His residues. Besides, the presence of a great amount of sodium ions sur-

rounding the enzyme molecule for balancing its overall negative charge may significantly increase the ionic strength in the surrounding.

The activation energies presented in Table 1 appeared to support the above kinetic data, being more related to the K_m rather than the V_{max}. This is reasonable and suggests that as compared to Brij 52, CTAB induced a more unfavorable binding between the enzyme and the substrate, thereby elevating the energy barrier of the enzymatic reaction and hence lowering the enzyme activity.

With regard to thermostability, our data show that tyrosinase, whether native or treated with surfactants, followed the classical first-order deactivation kinetics. A comparison of both the half-lives (Fig. 4) and the first-order deactivation rate constants (Table 2) indicates that the enzyme was stabilized by Brij 52 while being destabilized by either AOT or CTAB. This result seems to be supported by the optimum temperature data (Table 1) and the ability of different enzyme forms to retain activity at high reaction temperatures (Fig. 2), as mentioned in the result section. This is also supported by the lower activation energy for thermal deactivation of the enzyme incubated with either AOT or CTAB, as compared to that for native enzyme (Table 2). However, the low activation energy for deactivation of tyrosinase incubated with Brij 52 is not expected.

Our thermostability data obtained in aqueous solutions containing AOT and CTAB are in agreement with the results obtained with tyrosinase in both AOT and CTAB reversed micelles. Our previous work demonstrated that the same enzyme was much more unstable in both types of reversed micelles than in aqueous solution [3]. This suggests that either the high concentration of sodium ions or the CTAB layer surrounding the enzyme molecules was without effect on stabilizing the enzyme. Instead, Brij 52 appeared to be more effective in protecting the enzyme from thermoinactivation.

Overall, our results with AOT and CTAB-treated tyrosinases in aqueous solution agreed with our previous results obtained in reversed micelles in that:

(1) superactivity was achieved both in AOT-containing aqueous buffer and in AOT-containing reversed micelles, thereby reinforcing the activation effect of the anionic AOT on mushroom tyrosinase;
(2) a lower enzyme activity was observed both in CTAB-containing aqueous buffer and in CTAB-containing reversed micelles as compared to that in aqueous phosphate buffer without any surfactant, thus confirming the deactivation effect of the cationic CTAB on the enzyme;
(3) both AOT and CTAB destabilized the enzyme either in aqueous solution or in reversed micelles, and the destabilization effect of CTAB was more serious.

It has been reported that tyrosinase from different sources exists in either an active or a latent form or both, and that the activation of the enzyme by SDS is related to a conformational change from the latent enzyme to its active isoform [13]. The new conformation is more open or extended that facilitates catalysis and also makes the enzyme more vulnerable [12]. This can be used to explain the observation with AOT-treated tyrosinase in our study. In fact, the same phenomenon of "higher activity with lower stability" has been previously reported for both mushroom tyrosinase [11] and broad bean tyrosinase [10] treated with SDS.

CTAB showed both inactivation and destabilization effects on mushroom tyrosinase. It may follow the same kinetic mechanism of the same enzyme exposed to the cationic surfactant cetylpyridinium chloride (CPC) [15]: the enzyme undergoes a quick reversible binding with CPC and then a slow irreversible inactivation.

Interestingly, our experiments have demonstrated that mushroom tyrosinase can be both activated and stabilized by the non-ionic surfactant Brij 52. Mechanism for such activation and stabilization is worth further investigation.

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