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Effects of mixed reverse micellar structure on stability and activity of yeast alcohol dehydrogenase

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Abstract

The effects of Brij30 concentration on the stabilities and activities of yeast alcohol dehydrogenase (YADH) at various ω_0 values have been studied. Generally, the residual activity of YADH decreased rapidly first and then approached to a steady value. By the addition of Brij30, it was found that the stability of YADH could be enhanced significantly at $\omega_0 = 10$. However, at $\omega_0 = 20$, the stability of YADH was not remarkably improved and under some conditions was even lower than that in the absence of Brij30. By the investigation on the hydrodynamic diameter of mixed reverse micelles and its distribution via dynamic light scattering, it was suggested that the structure of mixed reverse micelles and the stability of YADH were determined by four important factors, including the surface charge density, bound water, reverse micellar size, and the entrapment of water by hydrophilic–hydrophilic interaction of Aerosol OT (AOT) and Brij30. The effects of these four factors on the stability of YADH at various ω_0 values and Brij30 concentration have been discussed in detail. When they were reduced, the stability of YADH might be improved. In addition, it was found that the activity of YADH in AOT/Brij30 mixed reverse micelles might be enhanced at appropriate Brij30 concentrations and ω_0 values. According to the hydrodynamic diameter of mixed reverse micelles and its distribution, three main factors were suggested. They were the hydrophobic and electrostatic interactions between enzyme and surfactants, the reverse micellar size, and the bound degree of water molecules. An optimal reverse micellar size and the reductions of the other two factors would lead to the enhancement of enzyme activity.

Keywords: Yeast alcohol dehydrogenase; Aerosol OT; Brij30; Reverse micelles; Stability; Hydrodynamic diameter; Dynamic light scattering

1. Introduction

Reverse micelles have received great attention during the past two decades because of their potential applications in the recovery and separation of proteins and in the enzymatic reactions in organic media [1–6]. Among the surfactants capable of forming reverse micelles, Aerosol OT (AOT) is of particularly interest because of its ability to form stable aggregates in a variety of organic solvents with relatively large amounts of water. However, the denaturation or deactivation of proteins and enzymes due to the hydrophobic and electrostatic interactions with AOT is a severe problem in the practical application of reverse micelles. In addition to the use of bile salt [7–9], one promising approach to overcome this problem is to reduce the surface charge density by modifying the reverse micellar interface via the addition of nonionic surfactants [10–19]. In general, it was found that the stabilities of enzymes in reverse micelles could be significantly improved by this approach [9,11,14,18].

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Alcohol dehydrogenase which catalyzes the oxidation of alcohols and the reduction of carbonyl compounds, such as aldehydes and ketones has received considerable attention because of its potential applications in the production of various starting materials and intermediates in chemical industry, the synthesis of chiral compounds, biosensors, and the regeneration of coenzymes NAD(P) and NAD(P)H [20–24]. Unfortunately, its stability in AOT reverse micelles was poor due to the solubilization process and to the hydrophobic and electrostatic interactions with AOT [25,26]. Some studies of circular dichroism (CD) and electron paramagnetic resonance (EPR) also showed that the conformation of horse liver alcohol dehydrogenase (HLADH) in AOT reverse micelles was altered and suggested that AOT molecules might interact with HLADH via adsorption and active-site contamination [27,28]. Only few efforts have been made to overcome this problem [9], restricting their practical applications. It is necessary and attractive to improve the stability of alcohol dehydrogenase in AOT reverse micelles.

In this work, the utilization of nonionic surfactant Brij30 to improve the stability of yeast alcohol dehydrogenase (YADH) in AOT reverse micelles was tried. It was found that the result was quite different from those reported previously and might concern with the structure of mixed reverse micelles. By the technique of dynamic light scattering (DLS), the hydrodynamic diameter (HD) distribution of AOT/Brij30 mixed reverse micelles which reflected some structural information but received no attention until now were investigated and used to reasonably account for the effect of Brij30 concentration on the activity and stability of YADH.

2. Materials and methods

2.1. Materials

Crystallized and lyophilized alcohol dehydrogenase (EC1.1.1.1) from baker's yeast (No. A-3263), β-nicotinamide adenine dinucleotide, reduced form (NADH, N-8129), sodium di-2-ethylhexylsulfosuccinate (AOT), and polyoxyethylene (4) lauryl ether (Brij30) were purchased from Sigma. *Iso*-octane, tris(hydroxymethyl)aminomethane (Tris), and hydrochloric acid were the guaranteed reagents of E. Merck (Darmstadt). Methyl ethyl ketone (MEK) used as the substrate was an analytical grade reagent of Ferak (Germany). Reagent-grade water produced by Milli-Q SP Ultra-Pure-Water Purification System of Nihon Millipore Ltd. (Tokyo) was used throughout this work.

2.2. Activity and stability assay of YADH

The YADH-entrapped reverse micellar solution was prepared by directly injecting appropriate amount of aqueous buffer containing YADH and NADH to the AOT *iso*-octane solution and mixing by a vortex. The activity of YADH was determined by measuring the initial reduction rate of MEK by YADH following the decrease of NADH concentration at 340 nm with a Hitachi U-3210 spectrophotometer. For each assay, appropriate amount of MEK was added to the reverse micellar solution and mixed to start the reaction. Then, the reaction mixture was placed in a capped-quartz cell without stirring for the measurement of reaction rate. All solutions were kept at 25 °C during operation. The cuvette block of spectrophotometer was also thermostated by a circulating water bath.

To investigate the stability of YADH in reverse micelles, a series of the same reverse micellar solutions containing YADH and NADH were tightly stopped in glass tubes and incubated in a water bath at 25 °C. After each specified incubation time, one sample was taken for the assay of YADH activity.

In this study, the degeneration of NADH was negligible in the period examined via the observation of its absorbance at 340 nm. The concentrations of AOT and MEK were based on the overall volume of reverse micellar solution. Since YADH and NADH were present in water pools, their concentrations were referred to the volume of aqueous buffer added in the reverse micellar solution. The concentrations of AOT, MEK, NADH, and YADH were fixed at 0.1 M, 0.05 M, 1.0 mM, and 0.1 mg/ml, respectively. The aqueous buffer used throughout this work was the Tris–HCl buffer (0.1 M, pH 8.1). The ω_0 value was defined as the molar ratio of water to AOT. The activity was calculated by a least-square method using the data recorded within the early several minutes, depending on the reaction rate.

2.3. Determination of hydrodynamic diameter distribution of reverse micelles

The HD distribution of reverse micelles, based on the intensity distribution, was measured by DLS using a commercial Malvern Autosizer 4700 spectrometer equipped with an Ar ion laser operating at 488 nm. Analysis of the correlation function, measured at $\theta = 90^\circ$ was carried out using the CONTIN algorithm.

3. Results and discussion

3.1. Hydrodynamic diameter distribution of mixed reverse micelles

As have been known, the properties of enzymes in reverse micelles depend strongly on the water content which affects the size of reverse micelles and the physical state of water. So, it is necessary to establish the fundamental information of mixed reverse micelles before the assay of the stability and activity of YADH. Fig. 1 shows the HD distribution of AOT reverse micelles at various ω_0 values. It was found that the HD of AOT reverse micelles was essentially monodispersed and increased with the increase of ω_0 value when $\omega_0 > 10$. However, the HD distributions at $\omega_0 = 7$ and 10 were broad. This might be due to the water content was too low to provide sufficient bound water because the viscosity of bound water was larger than that of free water [29]. Accordingly, it could be suggested that the maximum bound water required for AOT reverse micelles was located in the ω_0 range of 10–12. The ω_0 range was approximately consistent with those proposed previously [5]. Slight deviation might be resulted from the presence of buffer agents.

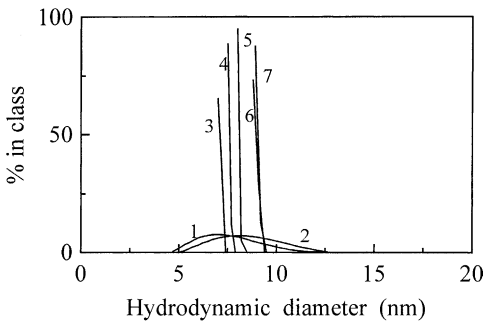

Fig. 1. Hydrodynamic diameter distributions of AOT reverse micelles at various ω_0 values; $\omega_0 = 7$ (1), 10 (2), 12 (3), 14 (4), 16 (5), 18 (6), 20 (7).

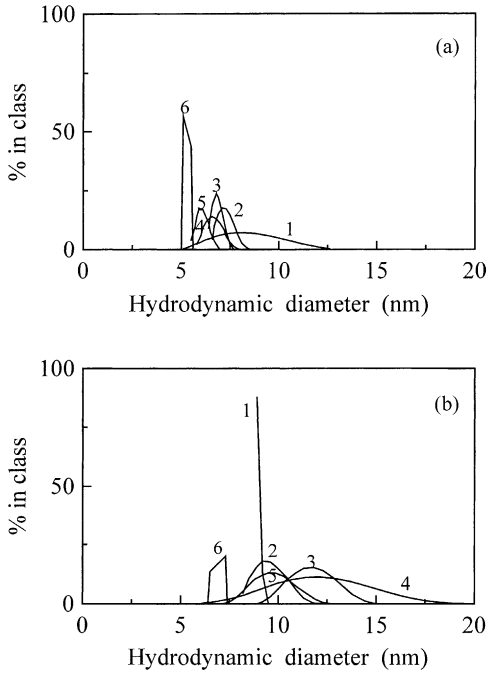

Fig. 2. Hydrodynamic diameter distributions of AOT/Brij30 mixed reverse micelles at various Brij30 concentrations. (a) $\omega_0 = 10$, (b) $\omega_0 = 20$; [Brij30] = 0 M (1), [Brij30] = 0.025 M (2), [Brij30] = 0.05 M (3), [Brij30] = 0.10 M (4), [Brij30] = 0.20 M (5), [Brij30] = 0.40 M (6).

The HD distributions of AOT/Brij30 mixed reverse micelles at $\omega_0 = 10$ and 20 were illustrated in Fig. 2. At $\omega_0 = 10$, with the increase of Brij30 concentration from 0.025 to 0.40 M, the HD of mixed reverse micelles decreased gradually while its distribution became narrow first, then abruptly became broad at [Brij30] = 0.1 M, and became narrow again when [Brij30] $\geq$ 0.2 M. At $\omega_0 = 20$, as Brij30 concentration increased, the HD of mixed reverse micelles increased and its distribution became broad in the Brij30 concentration range of 0.025–0.10 M. However, when [Brij30] $\geq$ 0.2 M, the HD decreased rapidly and its distribution became narrow again. These discrepant phenomena at $\omega_0 = 10$ and 20 have not been mentioned previously. They were interesting and could be explained as follows.

As mentioned above, the water content at $\omega_0 = 10$ was essentially less than the maximum bound water required for AOT. So, the addition of Brij30 might result in the reorganization of reverse micelles. The incorporation of Brij30 decreased the repulsive force among the head group of AOT surfactant and hence caused the reduction in the size of the mixed reverse micelles newly formed [2]. Furthermore, the maximum bound water required for nonionic Brij30 should be less than that for anionic AOT. So, with the increase in the molar ratio of Brij30/AOT, the maximum bound water required for the mixed reverse micelles would decrease and led to the HD distribution of mixed reverse micelles become narrow. When [Brij30] $\geq$ 0.1 M, the HD of mixed reverse micelles also followed a descending trend with the increase of Brij30 concentration. However, its distribution abruptly became broad at [Brij30] = 0.1 M and became narrow again when [Brij30] $\geq$ 0.2 M. This implied another factor influencing the HD distribution of mixed reverse micelles should be considered. Ogino et al. [30] investigated the mixed surfactant system of anionic (sodium dodecyl sulfate, SDS) and nonionic (alkyl poly(oxyethylene) ether; $C_mH_{2m+1}O(CH_2CH_2O)_{20}H$, $m = 16$ and 18) surfactants and proposed a hydration model. They suggested that the water molecules would be trapped between the oxygen atoms of the hydrophilic group of SDS and the ethylene oxide in the nonionic surfactant molecules. When equal moles of SDS and alkyl poly(oxyethylene) ether were used, the hydrophilic–hydrophilic interaction in the anionic–nonionic mixed surfactant system reached the maximum. According to their opinion, it also could be suggested that the entrapment of water molecules by AOT and Brij30 molecules via the hydrophilic–hydrophilic interaction was particularly significant at [Brij30] = 0.1 M. This counteracted the reduction of bound water due to the increase in the Brij30/AOT molar ratio of mixed reverse micelles, and led to a broader HD distribution.

At $\omega_0 = 20$, the water content was more than the maximum bound water for AOT. It could be imagined that the addition of Brij30 did not result in the recognization of AOT reverse micelles in the Brij30 concentration range of 0.025–0.10 M. Under this condition, the Brij30 molecules might be solubilized in the AOT surfactant head group region and resulted in the expansion of reverse micelles [16]. As for the broadened HD distribution, it could be due to the increase of total bound water resulted from the addition of Brij30 and the entrapment of water molecules by AOT and Brij30 molecules via the hydrophilic–hydrophilic interaction. When [Brij30] $\geq$ 0.2 M, the HD of mixed reverse micelles decreased abruptly and its distribution became narrow again. Since the HD distribution was quite broad at [Brij30] = 0.1 M, this phenomenon observed at [Brij30] $\geq$ 0.2 M could be due to that the water content was much less than the maximum bound water required for both AOT and Brij30, and the reorganization of reverse micelles must have occurred. With the increase of Brij30 concentration, the HD of mixed reverse micelles decreased and its distribution became narrow. Like that at $\omega_0 = 10$, this could be attributed to the reduction in bound water and the repulsive force among the head group of AOT surfactant for the reorganized reverse micelles.

Fig. 3 illustrates the dependences of the ratios of the mean hydrodynamic diameters (MHD) for AOT/Brij30 mixed reverse micelles to those for AOT reverse micelles on Brij30 concentration at various ω_0 values. Two contrary tendencies could be clearly observed. With the increase of Brij30 concentration, the ratio increased first and then decreased when the water content was above the maximum bound water required for AOT ($\omega_0 = 12$–20); however, the ratio decreased when the water content was less than the maximum bound water required for AOT ($\omega_0 = 7$ and 10). This indicated the addition of Brij30 had different influences on the structure of AOT/Brij30 mixed

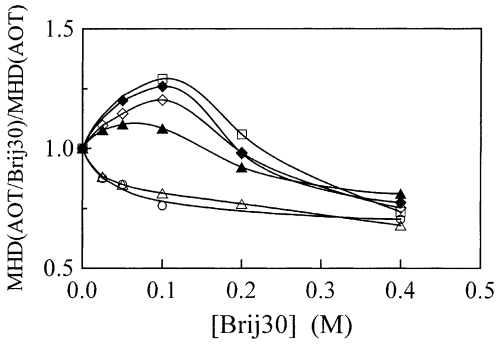

Fig. 3. Dependences of the ratios of the mean hydrodynamic diameters for AOT/Brij30 mixed reverse micelles and those for AOT reverse micelles on Brij30 concentrations at various ω_0 values; $\omega_0 = 7$ (○), 10 (△), 12 (▲), 14 (◇), 16 (◆), 20 (□).

reverse micelles and the physical state of water at different water contents. In addition, it was noted that the ratios were weakly dependent on the ω_0 values at a high Brij30 concentration of 0.4 M. The disordered relationship might be caused by the experimental errors.

The above phenomena about the AOT/Brij30 mixed reverse micelles were interesting and would be helpful to describe the stability and activity of YADH in the AOT/Brij30 mixed reverse micelles as investigated below.

3.2. Stability of YADH in mixed reverse micelles

Fig. 4a shows the time courses of the residual activities of YADH at $\omega_0 = 10$ and various Brij30 concentrations. In general, the residual activity of YADH decreased rapidly first and then approached to a steady value after 5–30 h, depending on Brij30 concentration. It was evident that the steady residual activity

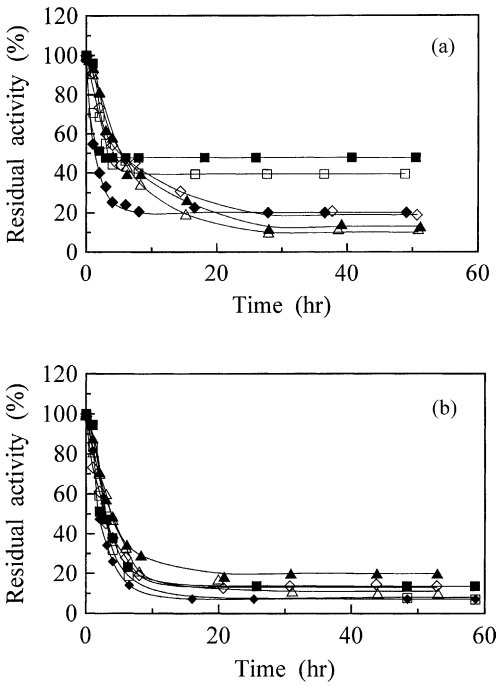

Fig. 4. Time courses of YADH residual activities in AOT/Brij30 mixed reverse micelles at various Brij30 concentrations; (a) $\omega_0 = 10$, (b) $\omega_0 = 20$; [Brij30] = 0 M (△), [Brij30] = 0.025 M (▲), [Brij30] = 0.05 M (◇), [Brij30] = 0.10 M (◆), [Brij30] = 0.20 M (□), [Brij30] = 0.40 M (■).

of YADH was enhanced significantly by the addition of Brij30, and the enhancement increased with the increase of Brij30 concentration. When [Brij30] = 0.4 M, the steady residual activity of YADH could be raised up to 48%, much higher than that in the absence of Brij30 (10%). This indicated the addition of nonionic Brij30 was indeed helpful to improve the stability of YADH in AOT reverse micelles. This result was in agreement with the expectation and could be attributed to the reduction in the surface charge density of reverse micelles. Moreover, previous studies indicated that the enzyme had higher stability in smaller reverse micelles because of the immobilization effect [31]. Since the HD of AOT/Brij30 mixed reverse micelles decreased with increasing Brij30 concentration at $\omega_0 = 10$, the reduction in reverse micellar size due to the addition of Brij30 could be another reason for the enhancement of YADH stability in mixed reverse micelles.

It was noteworthy that the initial deactivation rate of YADH decreased slightly with the increase of Brij30 concentration when [Brij30] < 0.1 M. However, the initial deactivation rate increased abruptly at [Brij30] = 0.1 M and decreased again when [Brij30] ≥ 0.2 M. Since the surface charge density decreased with the increase of Brij30 concentration and the initial deactivation did not change the ascending tendency of the steady residual activity of YADH, the increase in the initial deactivation rate of YADH might be concerned with its solubilization in reverse micelles. It is known that an enzyme has many hydrophilic groups on its surface and appropriate amount of bound water to stabilize itself is necessary. As mentioned above, the entrapment of water molecules by AOT and Brij30 molecules via the hydrophilic–hydrophilic interaction might be particularly significant at [Brij30] = 0.1 M and $\omega_0 = 10$. The increase in the water amount entrapped by surfactants might snatch the bound water required for the stabilization of YADH and resulted in the rapid deactivation of YADH in the early period. At [Brij30] = 0.2 and 0.4 M, the water content entrapped by AOT and Brij30 via the hydrophilic–hydrophilic interaction was less than that at [Brij30] = 0.1 M and hence the initial deactivation of YADH was not so rapid.

The time courses of the residual activities of YADH at $\omega_0 = 20$ and various Brij30 concentrations were shown in Fig. 4b. Similar to those observed at $\omega_0 = 10$, the residual activity of YADH decreased

rapidly first and then approached to a steady value after 15–20 h. However, the effect of Brij30 concentration on the stability of YADH in mixed reverse micelles was not so significant as that at $\omega_0 = 10$. As Brij30 concentration increased, the steady residual activity of YADH increased first, then decreased when [Brij30] $\geq$ 0.05 M, and increased again when [Brij30] $\geq$ 0.2 M. At [Brij30] = 0.1 and 0.2 M, the steady residual activities of YADH were even lower than that in the absence of Brij30. This phenomenon was out of the expectation that the stability of YADH could be improved by the addition of Brij30. It implied that at least two contrary factors were present simultaneously in the system examined. As expected, the addition of Brij30 might reduce the surface charge density of reverse micelles and improve the enzyme stability. However, the addition of Brij30 at $\omega_0 = 20$ also resulted in the expansion of reverse micelles except when [Brij30] $\geq$ 0.2 M. The increase of reverse micellar size would lead to the reduction in enzyme stability due to the lack of immobilization effect [31]. When [Brij30] $\geq$ 0.2 M, the mixed reverse micelles became smaller due to reorganization and hence the steady residual activity of YADH increased again.

According to the above investigations, it could be concluded that the effect of Brij30 concentration on the stability of YADH in AOT/Brij30 mixed reverse micelles was concerned with the surface charge density, bound water, reverse micellar size, and the hydrophilic–hydrophilic interaction of AOT and Brij30. The residual activity of YADH could be enhanced when these factors were reduced although they were opposite under some conditions.

3.3. Activity of YADH in mixed reverse micelles

Fig. 5a shows the variations of the initial activities of YADH in AOT/Brij30 mixed reverse micelles with Brij30 concentration at $\omega_0 = 10$ and 20. At $\omega_0 = 10$, with the increase of Brij30 concentration, the initial activity of YADH increased first as expected, but then gradually decreased down to a value even lower than that in the absence of Brij30 after reaching a maximum at [Brij30] = 0.025 M. It is known that Brij30 is a nonionic surfactant and its interaction with enzyme should be weaker compared to the anionic surfactant AOT. The addition of Brij30 might decrease the contact of enzyme and AOT. Thus, the increased activity at low

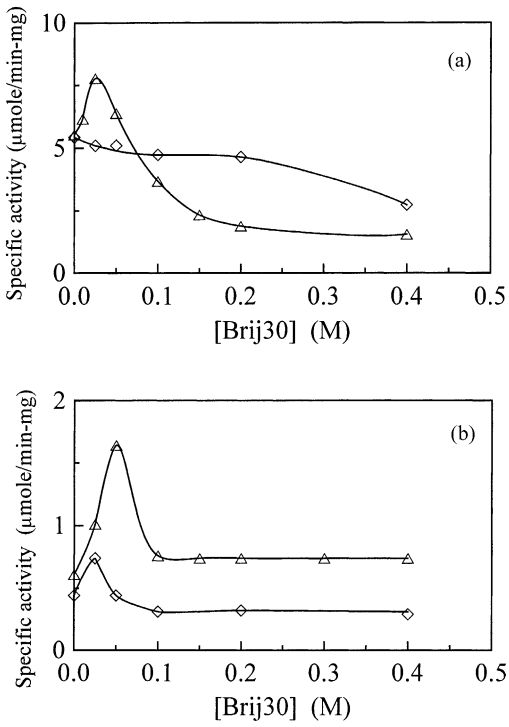

Fig. 5. Dependences of YADH activities in AOT/Brij30 mixed reverse micelles on Brij30 concentrations; (a) initial activity, (b) steady residual activity; $\omega_0 = 10$ (△) and 20 (◇).

Brij30 concentrations might be referred to the suppression of hydrophobic and electrostatic interactions between enzyme and surfactants due to the addition of Brij30. However, it is known the activity of enzyme in reverse micelles usually reaches a maximum when the size of reverse micelles fits the enzyme. According to our previous work [32], the optimal ω_0 value (i.e. an index of reverse micellar size) for the activity of YADH in AOT reverse micelles was around 10. Therefore, although the stability was raised, the reduction in size due to the addition of Brij30 at $\omega_0 = 10$ as mentioned above might be one reason why the initial activity of YADH decreased when [Brij30] > 0.025 M. Furthermore, it is known that the enzyme activity also depends on the physical state of water molecules in reverse micelles. When water molecules were strongly bound by surfactant molecules, the activity of enzyme would be reduced. According to the above investigations on HD distribution, it has been shown that the bound degree of water molecules decreased first, then increased at [Brij30] = 0.1 M, and finally

decreased again with the increase of Brij30 concentration. Thus, the bound degree of water molecules in AOT/Brij30 mixed reverse micelles might be another factor to affect the YADH activity. The bound degree of water molecules might be affected by the surface charge density of reverse micelles and the entrapment of water by hydrophilic–hydrophilic interaction of AOT and Brij30.

The phenomenon at $\omega_0 = 20$ was different from that at $\omega_0 = 10$. The initial activity of YADH decreased with increasing Brij30 concentration. No improvement in enzyme activity was observed. As illustrated in Fig. 2b, the HD of reverse micelles increased and its distribution became broad when Brij30 concentration was increased up to 0.1 M. Thus, for the initial activity of YADH in this concentration range of Brij30, it was revealed that both the increases in the revere micellar size and the bound degree of water molecules were over the suppression of hydrophobic and electrostatic interactions between enzyme and surfactants. This probably was the reason why the initial activity of YADH decreased. Although the HD of mixed reverse micelles decreased and its distribution became narrow after reorganization when [Brij30] $\geq$ 0.2 M, the initial activity of YADH remained the descending trend. Because the distribution was still broad and the HD of mixed reverse micelles was even smaller than that in the absence of Brij30 at $\omega_0 = 20$, the descending trend at [Brij30] $\geq$ 0.2 M might be due to the combined effect of three main factors on the reorganized reverse micelles: the reverse micellar size, bound degree of water molecules, and the suppression of hydrophobic and electrostatic interactions between enzyme and surfactants.

The dependencies of the steady residual activities of YADH on Brij30 concentration in AOT/Brij30 mixed reverse micelles at $\omega_0 = 10$ and 20 were shown in Fig. 5b. It was found that the steady residual activities at $\omega_0 = 10$ were higher than that in the absence of Brij30 over the whole concentration range of Brij30 examined. Furthermore, with the increase of Brij30 concentration, the steady residual activity increased first, then decreased after reaching a maximum at [Brij30] = 0.05 M, and finally remained at a constant value when [Brij30] $\geq$ 0.1 M. The curve at $\omega_0 = 20$, exhibited a similar tendency except that the maximum occurred at [Brij30] = 0.025 M and the steady residual activities at [Brij30] $\geq$ 0.1 M were lower than that in the absence of Brij30. These phenomena could be interpreted just like the above discussion for the initial activity. Their differences with those for the initial activity might be due to the changes in the enzyme properties after partial deactivation. Anyhow, it could be concluded that the addition of appropriate amounts of Brij30 (<0.1 M) would be helpful for the enhancement of the steady residual activity of YADH in AOT reverse micelles. This is important for a long-term operation.

4. Conclusions

The stability of YADH in AOT/Brij30 mixed reverse micelles has been studied. In general, the residual activity of YADH decreased rapidly first and then approached to a steady value. At $\omega_0 = 10$, the stability of YADH was enhanced with the increase of Brij30 concentration as expected. However, at $\omega_0 = 20$, almost no significant improvement was observed and the stabilities under some conditions were even lower than that in the absence of Brij30. This phenomenon was quite different from those reported previously for other enzymes such as lipase and α-chymotrypsin [9,11,14,18]. By the investigation on the HD of mixed reverse micelles and its distribution, four important factors affecting the structure of mixed reverse micelles and the stability of YADH could be clarified. They were the surface charge density, bound water, reverse micellar size, and the entrapment of water by hydrophilic–hydrophilic interaction of AOT and Brij30. The reductions of these factors would favor the stability of YADH although they were opposite under some conditions. Furthermore, it was found that the activity of YADH in AOT/Brij30 mixed reverse micelles was affected by the hydrophobic and electrostatic interactions between enzyme and surfactants, the reverse micellar size, and the bound degree of water molecules. Except an optimal reverse micellar size was needed, the activity of YADH might be enhanced by the reductions of the other two factors.

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