# DEACTIVATION KINETICS OF YEAST ALCOHOL DEHYDROGENASE IN AEROSOL OT/ISOOCTANE REVERSE MICELLES

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***Key Words:*** Yeast Alcohol Dehydrogenase, Deactivation Kinetics, Reverse Micelles, AOT, Isooctane

**The deactivation kinetics of yeast alcohol dehydrogenase (YADH) in both aerosol OT/isooctane reverse micelles and aqueous buffer were studied. The YADH entrapped in reverse micelles could retain activity for above 24 hr although it was less stable than dissolved in aqueous buffer. Both the activity-time curves for the YADH in reverse micelles and in aqueous buffer exhibited a rather rapid exponential decay within the early 2 hr, followed by a slower exponential decay during the remaining period. A series-type enzyme deactivation model involving two first-order steps and one active intermediate was used to describe the deactivation behavior of YADH. The kinetic parameters of the deactivation rate equations were obtained by optimization method. In aqueous buffer, the deactivation rate of YADH exhibited a maximum around a Tris concentration of 0.1 mol·m$^{-3}$. The deactivation rate of YADH in reverse micelles was strongly dependent on Tris concentration and the molar ratio of water to surfactant ($\omega_0$). The residual activity percentage of the active intermediate increased with the increase of $\omega_0$ and Tris concentration, while both the rate constants for the first and second first-order deactivation steps decreased with the increase of Tris concentration.**

## Introduction

Reverse micelles can be used for the recovery and separation of proteins, and for performing the biocatalytic reactions in organic media. The investigation of reverse micelles has received great attention of the past two decades.[9, 13, 17, 18, 20, 22] Many authors have reported a decrease in enzymatic activity with time on incubation of enzymes in reverse micelles.[5, 6, 8, 14, 15, 25] Since the stability of enzyme is an important consideration in the feasibility of many biotechnological processes, the deactivation kinetics of enzyme in reverse micelles was emphasized in this study.

Alcohol dehydrogenase, which catalyzes the oxidation of alcohols and the reduction of carbonyl compounds such as aldehydes and ketones, can be used widely for the production of various starting materials and intermediates in chemical industry,[4, 6, 28] the synthesis of chiral compounds,[11, 12] and the regeneration of coenzymes NAD (P) and NAD (P)H.[11, 30] The studies of alcohol dehydrogenase in reverse micelles have been reported by several authors.[1, 14, 15, 19, 21, 24-27] However, almost all attention was focused on horse liver alcohol dehydrogenase (HLADH).[1, 14, 15, 19, 21, 24, 26, 27] Very few work was done on yeast alcohol dehydrogenase (YADH). Recently, Sarcar *et al.*[25] studied the activity and stability of YADH in AOT/isooctane reverse micelles by examining the oxidation of ethanol with NAD as coenzyme. Their experimental results indicated that the YADH incubated in $NAD^+$-containing reverse micelles became inactive in 30 min at 26°C, pH 8.1 and $\omega_0$ = 28. The poor stability of oligomeric proteins is a drawback in practice.

In this paper, the deactivation kinetics of the YADH incubated in NADH-containing AOT/isooctane reverse micelles was studied, and compared with that in aqueous buffer. Methyl ethyl ketone (MEK) was used as the substrate for the determination of enzyme activity. Tris was used for the preparation of buffer solution, in which NADH had better stability.[29] A deactivation kinetic model was proposed, according to which the effects of $\omega_0$ and Tris concentration on the deactivation rate of YADH could be analyzed.

## 1. Theoretical Consideration

Enzyme deactivations may be broadly classified into first-order and non-first-order processes. In general, a first-order model is sufficient to represent enzyme deactivation kinetics adequately. However, the non-first-order deactivation behavior was also often observed due to the complex nature of enzyme molecules.

$$E \xrightarrow{kd} E_d \tag{1}$$

where $E$ and $E_d$ denote the active and deactivated enzymes, respectively, and $k_d$ is the first-order deactivation rate constant. The deactivation rate equation can be expressed as

$$a = a_o \exp(-k_d t) \tag{2}$$

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where $a_o$ and $a$ represent the initial and residual activity of enzyme, respectively, and t is the incubation time of enzyme.

The non-first-order deactivation processes are usually described by sum-of-exponential type equations, which imply the existence of series-type enzyme deactivations.[10, 23] The most common series-type deactivation model is one involving two first-order steps and one active intermediate:[10, 23]

$$E \xrightarrow{k_1} E^* \xrightarrow{k_2} E_d \tag{3}$$

where $k_1$ and $k_2$ are the first-order deactivation rate constants, and $E^*$ denotes the active intermediate. Introducing the specific activity ratio of $E^*$ to $E$, $\beta$, the deactivation rate equation can be derived as[10, 23]

$$a_r = a / a_o = \left(1 + \frac{\beta k_1}{k_2 - k_1}\right)\exp(-k_1 t) + \left(\frac{-\beta k_1}{k_2 - k_1}\right)\exp(-k_2 t) \tag{4}$$

where $a_r$ is the ratio of residual activity to initial activity.

## 2. Experimental

### 2.1 Materials

Crystallized and lypophilized alcohol dehydrogenase (EC1.1.1.1) from Bakers yeast (No. A-3263), reduced form of $\beta$-nicotinamide adenine dinucleotide (NADH, N-8129), and sodium di-2-ethylhexylsulfosuccinate (aerosol OT, AOT) were purchased from Sigma Chemical Co. (St. Louis, MO). Isooctane, tris (hydroxymethyl) aminomethane (Tris), and the hydrochloric acid used to adjust the pH of Tris buffer were the guaranteed reagents of E. Merck (Darmstadt). Methyl ethyl ketone (MEK)was an analytical grade reagent of Ferak (West Berlin). 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 Kinetic Measurements in reverse micellar solution

The enzyme-entrapped reverse micellar solution was prepared by directly injecting appropriate amount of aqueous buffer containing YADH and NADH to 5 $cm^3$ AOT-isooctane solution and mixing by a vortex. To investigate the deactivation kinetics of YADH in reverse micelles, a series of the same reverse micellar solution containing enzyme and coenzyme were tightly stoppered in glass tubes and incubated in a water bath at 25°C. After each specified incubation time, one sample was taken for the measurement of enzyme activity.

The enzyme activity was determined by measuring the initial reduction rate of MEK by YADH following the decrease of NADH concentration at 340 nm with a Hitachi-330 spectrophotometer. For each measurement, 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 solution were kept at 25°C during operation. The cuvette

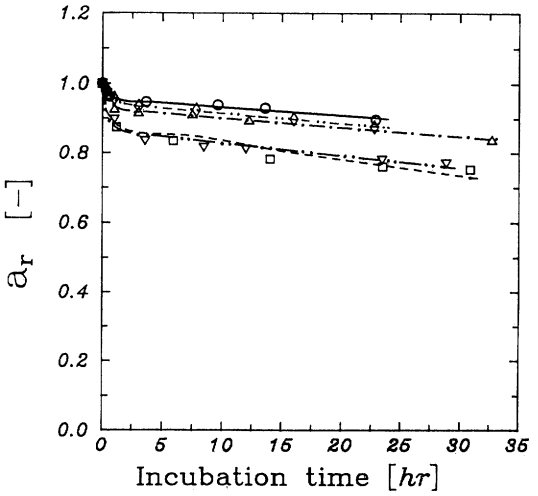



**Fig. 1** Variations of enzyme activity with incubation time in aqueous buffer at 25°C and pH 8.1.
Experimental data: [Tris] = 0.02 (○), 0.05 (▽), 0.10 (□), 0.15 (◇), 0.20 (△) mol $dm^{-3}$; calculated deactivation rate equation (Eq. (4)): [Tris] = 0.02 (——), 0.05 (– • •–), 0.10 (– – – –), 0.15 (– – • •), 0.20 (– • –) mol $dm^{-3}$, with $k_1$, $k_2$ and $\beta$ as indicated in Table 1; Condition for enzyme incubation: [NADH] = 1.0 mmol $dm^{-3}$, [YADH] = 0.5 mg $cm^{-3}$; Condition for activity measurement: [NADH] = 0.2 mmol $dm^{-3}$, [YADH] = 0.1 mg $cm^{-3}$, [MEK] = 0.1 mol $dm^{-3}$

block of spectrophotometer was also thermostated by a circulating water bath.

The concentration of substrate MEK was based on the overall volume of reverse micellar solution. Since enzyme and NADH were present in water pools, their concentrations were referred to the volume of aqueous buffer added in the reverse miocellar solution.

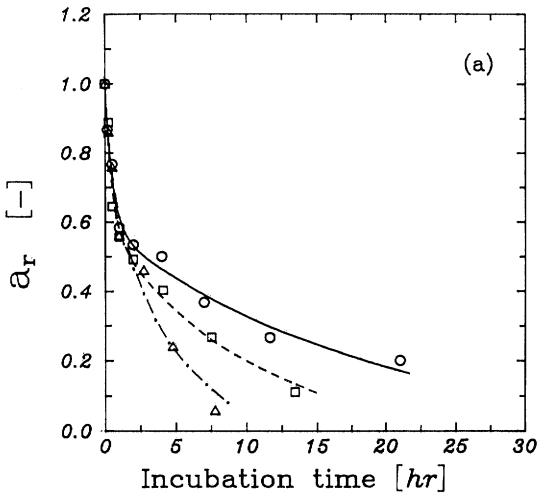
In this study, the concentrations of AOT, MEK, NADH, and YADH were fixed at 0.1 mol·$dm^{-3}$, 0.1 mol·$dm^{-3}$, 1.0 mmol·$dm^{-3}$, and 0.5 mg $cm^{-3}$, respectively. The pH of aqueous buffer used for the preparation of reverse micellar solution was constant at 8.1. The activity was calculated by a least-square method using the data recorded within the early 3-5 min, depending on the reaction rate.

### 2.3 Kinetic measurements in bulk aqueous buffer

The method for measuring the deactivation kinetics of YADH in bulk aqueous buffer was similar to that in reverse micellar solution. A series of the same Tris buffer solution containing YADH and NADH were incubated in a water bath at 25°C. After each specified incubation time, one sample was taken for the measurement of enzyme activity.

The enzyme activity was also determined by measuring the absorbance of NADH at 340 nm. The MEK was dissolved in the Tris buffer to prepare the substrate solution. For each measurement, the reaction was started by adding 1 $cm^3$ enzyme solution to 4 $cm^3$ substrate solution, both having the same Tris concentration.

All experiments were conducted at 25°C and pH 8.1. For the incubation of enzyme, the concentrations of NADH and YADH were fixed at 1.0 mmol·$dm^{-3}$ and 0.5



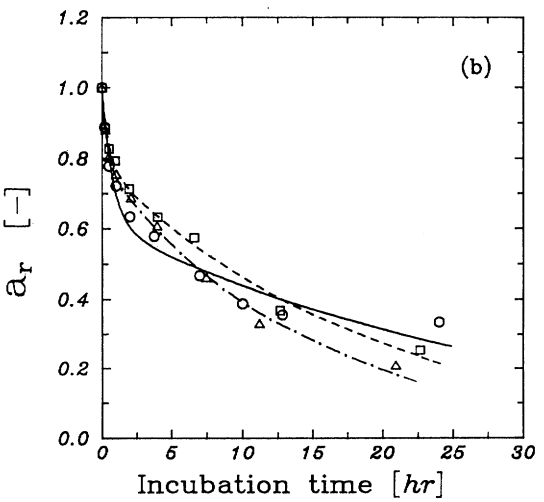
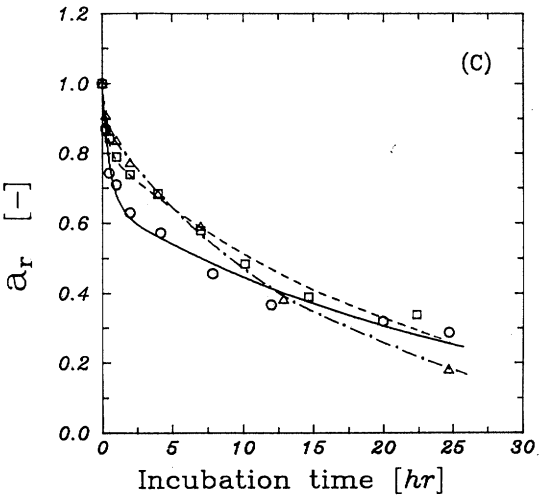




Fig. 2(a), (b)



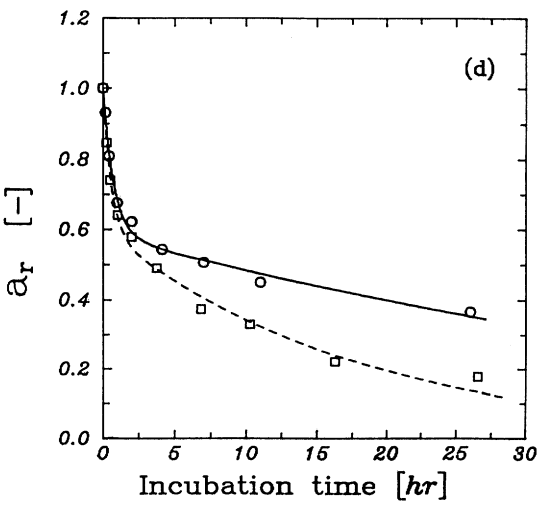




**Fig. 2** Variations of enzyme activity with incubation time in ATO / isooctane reverse micelles at 25°C. (a) $w_o$ = 10, (b) $w_o$ = 20, (c) $w_o$ = 25, (d) $w_o$ = 30, Experimental data: [Tris] = 0.02 (○), 0.10 (□), 0.20 (△), mol $dm^{-3}$; calculated deactivation rate equation (Eq. (4)): [Tris] = 0.02 (——), 0.10 (----), 0.20 (– • –) mol $dm^{-3}$, with $k_1$, $k_2$ and $\beta$ as indicated in Table 2; Condition for enzyme incubation: [AOT] = 0.1 mol $dm^{-3}$, [NADH] = 1.0 mmol $dm^{-3}$, [YADH] = 0.5 mg $cm^{-3}$, pH of aqueous buffer for preparing reverse micellar solution is 8.1; Condition for activity measurement: [MEK] = 0.1 mol $dm^{-3}$

mg $cm^{-3}$. While measuring the enzyme activity, the concentration of MEK, NADH and YADH were kept constant at 0.1 mol·$dm^{-3}$, 0.2 mmol·$dm^{-3}$ and 0.1 mg $cm^{-3}$, respectively.

## 3. Results and Discussion

### 3.1 Deactivation behavior of YADH

The deactivation behavior of YADH in aqueous buffer at various Tris concentrations was illustrated in a plot of $a_r$ vs. incubation time as shown in **Fig. 1**. The deactivation rate of YADH was slightly affected by Tris concentration, and a maximum deactivation rate seemed to appear around a Tris concentration of 0.1 mol·$dm^{-3}$. The YADH incubated in aqueous buffer retained at least 70% activity after 30hr.

The deactivation behavior of YADH in reverse micelles at various $\omega_o$ and Tris concentration was shown in **Fig. 2**. It was found that the deactivation rate of YADH in reverse micelles was strongly dependent on both $\omega_o$ and Tris concentration, but no consisitent relationship could be obtained for $\omega_o$ or for Tris concentration. Comparing with Fig. 1, it can be clearly seen that the YADH entrapped in reverse micelles was less stable than dissolved in aqueous buffer. However, the YADH incubated in reverse micelles could retain activity for above 24 hr. This result was much better than that observed by Sarcar *et al.*[25]

### 3.2 Deactivation kinetic model

As can be seen from Figs. 1 and 2, both the deactivation curves for the YADH in aqueous buffer and in reverse micelles did not follow a first-order deactivation model. That is, Eq. (2) cannot fit well with the experimental data. In fact, the deactivation curves were found to exhibit a rather rapid exponential decay within the early 2 hr, followed by a slower exponential decay during the remaining period. This revealed that the deactivation of YADH

**Table 1** Kinetic parameters of deactivation rate equation for YADH in aqueous buffer at 25°C

| [Tris] [mol dm$^{-3}$] | $k_1$ [hr$^{-1}$] | $k_2$ [hr$^{-1}$] | $\beta$ [-] | $a_o$ [$\mu$mol·min$^{-1}$mg$^{-1}$] |
|---|---|---|---|---|
| 0.02 | 1.89 | 0.00251 | 0.955 | 0.0545 |
| 0.05 | 2.17 | 0.00448 | 0.864 | 0.0515 |
| 0.10 | 3.09 | 0.00624 | 0.884 | 0.0509 |
| 0.15 | 1.53 | 0.00333 | 0.945 | 0.0476 |
| 0.20 | 2.05 | 0.00305 | 0.927 | 0.0528 |

**Table 2** Kinetic parameters of deactivation rate equation for YADH in AOT / isooctane reverse micelles at 25°C

| $w_o$ [-] | [Tris] [mol dm$^{-3}$] | $k_1$ [hr$^{-1}$] | $k_2$ [hr$^{-1}$] | $\beta$ [-] | $a_o$ [$\mu$mol·min$^{-1}$mg$^{-1}$] |
|---|---|---|---|---|---|
| 10 | 0.02 | 1.87 | 0.0584 | 0.567 | 0.0699 |
| | 0.10 | 2.18 | 0.110 | 0.565 | 0.0402 |
| | 0.20 | 2.68 | 0.248 | 0.705 | 0.0560 |
| 20 | 0.02 | 1.31 | 0.0340 | 0.600 | 0.0510 |
| | 0.10 | 2.04 | 0.0530 | 0.764 | 0.0409 |
| | 0.20 | 2.63 | 0.0695 | 0.765 | 0.0475 |
| 25 | 0.02 | 1.90 | 0.0381 | 0.640 | 0.0445 |
| | 0.10 | 2.98 | 0.0450 | 0.793 | 0.0380 |
| | 0.20 | 4.42 | 0.0632 | 0.872 | 0.0429 |
| 30 | 0.02 | 1.34 | 0.0194 | 0.578 | 0.0431 |
| | 0.10 | 1.65 | 0.0553 | 0.573 | 0.0375 |

could follow a series-type enzyme deactivation model. It had been reported that the experimental data for the deactivation of HLADH in aqueous buffer containing 5 mmol·dm$^{-3}$ adenosine monophosphate at pH 9 and 60°C, which were obtained by Gorisch and Schneider,[7] could be well fitted by a sum-of-exponential type equation like Eq. (4) with $k_1$, $k_2$ and $\beta$ being 0.047 min$^{-1}$, 0.008 min$^{-1}$ and 0.89.[23] Therefore, in this study we used Eq. (4) to describe the deactivation behavior of YADH.

By Rosenbrock optimization method,[2] the experimental data for the deactivation of YADH in aqueous buffer and in reverse micelles could be fitted with Eq. (4). The values of the three parameters $k_1$, $k_2$ and $\beta$ for various conditions are listed in **Tables 1** and **2**. The averate errors are 1.0 % for aqueous buffer system and 4.8 % for reverse micellar system. The calculated deactivation curves according to Eq. (4) are also shown in Figs. 1 and 2.

According to Tables 1 and 2, the following information could be obtained:

1. For both systems of aqueous buffer and reverse micelles, the values of $k_1$ are much larger than those of $k_2$.
2. The values of $k_1$ for aqueous buffer system are close to those for reverse micellar system, whereas the values of $k_2$ for aqueous buffer are much smaller than those for reverse micellar system. This implies that the factors leading to the first-step deactivation of YADH may be the same for both systems of aqueous buffer and reverse micelles.
3. The values of $\beta$ for aqueous buffer system are in agreement with the data of Gorisch and Schneider,[7, 23] and are larger than those for reverse micellar system. Furthermore, except for the case of $\omega_o$ = 30, the values of $\beta$ for the reverse micellar system increase with the increase of $\omega_o$. This tendency could be due to the fact that the properties of water pool approach to those of bulk water with increasing water content although small differences may remain at relatively larger $\omega_o$.[17] For the case of $\omega_o$ = 30, the lower $\beta$ values may be because the water content is close to the limiting amount of soluble water, under such a condition the deactivation of enzyme by organic solvent or surfactant becomes significant.
4. In aqueous buffer, the values of $k_1$ and $k_2$ vary with the Tris concentration. For both deactivation rate constants, their maximum values appear around a Tris concentration of 0.1 mol·dm$^{-3}$.
5. In reverse micellar solutions, the values of $\beta$ increase with the increase of $\omega_o$ and Tris concentration. However, both the values of $k_1$ and $k_2$ also increase with the increase of Tris concentration at each $\omega_o$. This reveals that the active intermediate retains higher residual activity percentage at higher Tris concentration, whereas the active intermediate also lose their activities more rapidly in the meanwhile. This information seems to explain why in appearance the enzyme deactivation rate has no consistent relationship with $\omega_o$ or Tris concentration in reverse micellar system, particularly when $\omega_o$ = 20 and 25.

In addition, to obtain an idea about the activities of YADH in aqueous buffer and in reverse micelles, the initial activities of YADH are also indicated in Tables 1 and 2. The effects of $\omega_o$ and Tris concentration on the activity of YADH has been discussed in our other recent work.[3]

## Conclusion

Both the deactivation kinetics of YADH in AOT/isooctane reverse micelles and in aqueopus buffer have been studied. Although the YADH entrapped in reverse micelles was less stable than dissolved in aqueous buffer, it could retain activity for above 24 hr. This result was much better than that reported in literature. Both the activity-time curves for the YADH in aqueous buffer and in reverse micelles could be expressed by a superposition of two exponential curves, with the first-step deactivation rate being faster than the second one. A series-type enzyme deactivation model involving two first-order steps and one active intermediate has been used to describe satisfactorily the deactivation behavior of YADH. By optimization method, the parameters of the deactivation rate equation were obtaine. The deactivation rate of YADH in aqeous buffer exhibited a maximum around 0.1 mol·dm$^{-3}$ Tris. The deactivation rate of YADH in reverse micelles was strongly dependent on both $\omega_o$ and Tris concentration. The residual activity percentage of active intermediate increased with the increase of $\omega_o$ and Tris concentration, while both the first-order deactivation rate constants decreased with the increase of Tris concentration.

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E006-295, to which the authors wish to express their thanks.

**Nomenclature**

| | | | |
|---|---|---|---|
| $a$ | = | residual activity | [$\mu$mol min$^{-1}$mg$^{-1}$] |
| $a_o$ | = | initial activity | [$\mu$mol min$^{-1}$mg$^{-1}$] |
| $a_r$ | = | ratio of residual activity to initial activity | [-] |
| $E$ | = | active enzyme | |
| $E_d$ | = | deactivated enzyme | |
| $E^*$ | = | active intermediate | |
| $k_d$ | = | first-order deactivation rate constant | [hr$^{-1}$] |
| $k_1$ | = | first-order deactivation rate constant | [hr$^{-1}$] |
| $k_2$ | = | first-order deactivation rate constant | [hr$^{-1}$] |
| $t$ | = | incubation time | [hr] |
| [AOT] | = | AOT concentration | [mol·dm$^{-3}$] |
| [MEK] | = | MEK concentration | [mol·dm$^{-3}$] |
| [NADH] | = | NADH concentration | [mmol·dm$^{-3}$] |
| [Tris] | = | Tris concentration | [mol·dm$^{-3}$] |
| [YADH] | = | YADH concentration | [mg·dm$^{-3}$] |

**Greek letters**

| | | | |
|---|---|---|---|
| $\beta$ | = | specific activity ratio of $E^*$ to $E$ | [-] |
| $\omega_o$ | = | molar ratio of water to AOT | [-] |

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