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ORIGINAL PAPER

Thermal Stability of Immobilized Lipase from *Candida antarctica* in Glycerols with Various Water Contents at Elevated Temperatures

Takashi Kobayashi · Takemasa Matsuo · Yukitaka Kimura · Shuji Adachi

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Abstract The immobilized lipase from *Candida antarctica* (fraction B, CALB) was incubated in glycerols with various water contents at 80–100 °C to measure the residual activity as a function of time. The glycerol-containing water stabilized the immobilized CALB, especially at 30–60 wt% water contents. The thermal inactivation behaviors of the immobilized CALB were expressed by a model in which the free energy of activation for the inactivation of the immobilized lipase molecules obeyed a Gaussian distribution.

Keywords *Candida antarctica* fraction B lipase · Gaussian distribution · Glycerol · Immobilization · Thermal inactivation

Introduction

Lipase has been widely used in oil processing for esterification, hydrolysis or transesterification. The lipase-catalyzed reaction has the characteristic that the product can be easily obtained in a high yield with a high selectivity compared to a conventional chemical process because of its high regio- and stereoselectivities. In addition, the immobilized lipase is often used for an industrial process because of its easy removal from the reaction mixture. Therefore, attention has been paid to the syntheses of various functional esters, such as sugar and sugar alcohol esters, using immobilized lipase in organic solvents [1–3]. In order to effectively perform these reactions, optimization of the reaction conditions is required including the reaction temperature which governs the thermal stability of the immobilized lipase, the reaction rate, and the equilibrium conversion.

In this study, we focused on the thermal stability of immobilized lipase in glycerol, which is one of the most simple sugar alcohols and generally used as a stabilizer for enzymes. Lipases generally require a certain amount of water to exhibit their catalytic activities [4, 5]. Therefore, the thermal inactivation behaviors of immobilized lipase from *Candida antarctica* (fraction B, CALB), which has been widely used for the synthesis of various glyceryl esters, were evaluated in glycerols with various water contents. Especially, the inactivation of immobilized CALB in glycerols with low water contents at elevated temperatures was mainly examined because of the higher yield of an ester in glycerol with a lower water content and of the higher reaction rate at higher temperature.

Materials and Methods

Materials

Immobilized CALB (Chirazyme® L-2, cf.-C2) was purchased from Roche (Mannheim, Germany). Glycerol, 1-butanol, lauric acid, and other chemicals of analytical grade were from Wako Pure Chemical Industries (Osaka, Japan).

Thermal Treatment of Immobilized Lipase

Weighed amounts of immobilized CALB (50 mg) and glycerol (0.5 mL) containing distilled water were placed in

T. Kobayashi · T. Matsuo · Y. Kimura · S. Adachi (✉)
Division of Food Science and Biotechnology,
Graduate School of Agriculture, Kyoto University,
Sakyo-ku, Kyoto 606-8502, Japan
e-mail: adachi@kais.kyoto-u.ac.jp

T. Kobayashi
e-mail: tkoba@kais.kyoto-u.ac.jp

a glass vial. The water content of the glycerol was 2–100 wt%. The vial was then immersed in an oil bath at 80–100 °C. Five to eight vials were prepared for each treatment temperature. At appropriate intervals, a vial was removed from the bath to estimate the residual activity of the immobilized CALB.

Assay of Lipase Activity

A lipase that is inactivated at elevated temperature does not generally become active again at a lower temperature. Therefore, the residual activity of the immobilized CALB was evaluated by measuring the esterification rate of lauric acid with 1-butanol at a moderate temperature as follows: immobilized CALB dipped in glycerol was first thoroughly washed three times with acetone to remove the glycerol and then dried under vacuum to obtain the thermally treated CALB. Dry acetone (9.95 mL), lauric acid (100 mg, 0.5 mmol), and 1-butanol (37 mg, 0.5 mmol) were placed in a glass vial, and the mixture was kept at 50 °C for 5 min. The thermally treated CALB (25 mg) was then added to the mixture to start the esterification with shaking at 120 rpm. At appropriate intervals, the reaction mixture (10 μL) was sampled for HPLC analysis.

The concentration of 1-butyl laurate was determined using an LC-20AT*VP* HPLC (Shimadzu, Kyoto, Japan) connected to a CapCell Pak C18 column (4.6 × 150 mm, Shiseido, Tokyo, Japan) and an SPD-20AV*VP* UV photometer (Shimadzu; 220 nm). The eluent was a methanol/water mixture (95/5, v/v) at 1.0 mL/min.

Results and Discussion

Figure 1 shows the thermal inactivation processes of the immobilized CALB held in glycerols with 2–10 wt% water contents on a semi-logarithmic scale. The immobilized CALB was very stable at 80 °C in glycerol with any water content, indicating that the immobilized CALB can be used as an effective catalyst for the synthesis of glyceryl esters at that temperature.

However, the inactivation was significant at 90 °C or higher. The semi-logarithmic plots of the residual activity versus time did not give a straight line, and the thermal inactivation did not obey the first-order kinetics. Similar inactivation behaviors were reported for the immobilized α-chymotrypsin in buffer solutions [6] and for the immobilized CALB in primary alcohols [7], but they were well explained by the model which was based on the following basic idea. Enzyme molecules in free form have the same susceptibility to thermal inactivation, but their immobilization results from chemical modification of the molecules in various ways, thus the molecules would have

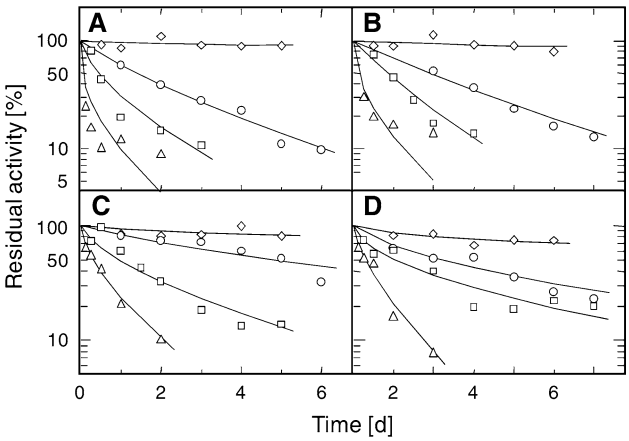

Fig. 1 Inactivation processes of the immobilized CALB in glycerols with (**a**) 2 wt%, (**b**) 5%, (**c**) 7.5% and (**d**) 10% water contents at (*open diamond*) 80 °C, (*open circles*) 90 °C, (*open squares*) 95 °C and (*open triangles*)100 °C. *Solid curves* are simulated ones

different thermal stabilities. The heterogeneity of the thermal stability among the enzyme molecules was expressed by a Gaussian distribution having a SD of σ for the free energy of activation, $\Delta G^{\ddagger}$, for the inactivation process, and the fraction of residual activity, C_e/C_{e0}, was formulated as a function of the incubation time t, by Eq. 1 [6]:

$$\frac{C_e}{C_{e0}} = \frac{RT}{\sqrt{2\pi}\sigma} \int_{-\infty}^{\infty} \exp\left(\frac{-R^2T^2(\ln k_d - \ln \bar{k}_d)^2}{2\sigma^2}\right) \times \exp(-k_d t)\mathrm{d}(\ln k_d) \quad (1)$$

where C_e is the concentration of the active enzyme, C_{e0} the initial C_e, R the gas constant, T the absolute temperature, k_d the rate constant of thermal inactivation for each enzyme molecule, and $\bar{k}_d$ is the k_d corresponding to the mean $\Delta G^{\ddagger}$.

This model was also applied to the inactivation of immobilized CALB in glycerol containing water. A set of two parameters, $\bar{k}_d$ and σ, were determined by a trial-and-error method to minimize the sum of the residual squares between the calculated and experimental results. The simulated results of the thermal inactivation could well express the experimental ones for every case as shown by the solid curves in Fig. 1. This fact reveals that the model is applicable to the thermal inactivation behavior of the immobilized CALB in glycerol containing water. The model suggests that the immobilized CALB retains its activity at a certain level even after a long incubation at a high temperature. The residual activity would be ascribed to the enzyme molecules tightly bound to the immobilization carrier and a having high thermal stability.

The temperature dependence of the $\bar{k}_d$ value could be expressed by the Arrhenius equation:

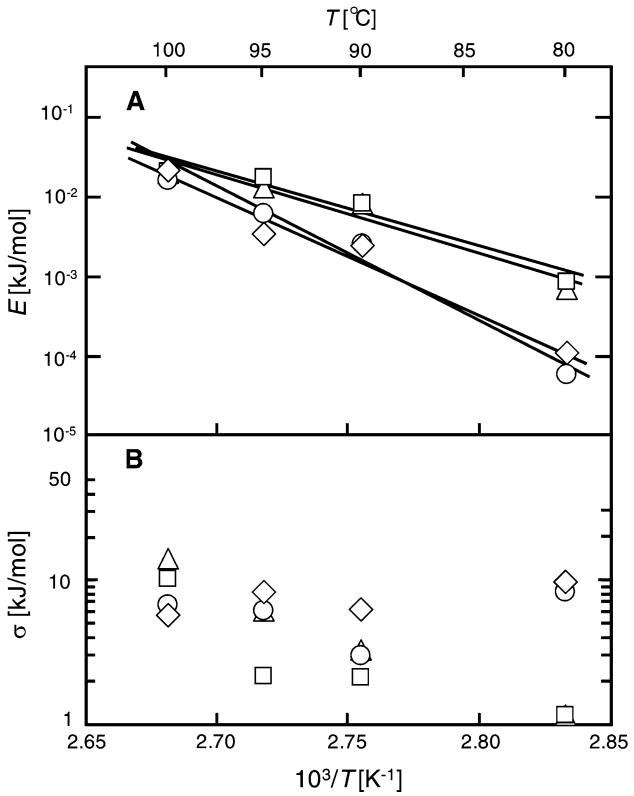

Fig. 2 Temperature dependences of (**a**) the rate constant corresponding to the mean free energy of activation, $\bar{k}_d$, and (**b**) the SD σ in thermal inactivation of the immobilized CALB in glycerols with (*open triangle*) 2 wt%, (*open square*) 5%, (*open circle*) 7.5% and (*open diamond*) 10% water contents

$$\ln \overline{k_d} = -\frac{E}{RT} + \ln k_0 \quad (2)$$

where E and k_0 are the activation energy and frequency factor, respectively, for thermal inactivation of the immobilized CALB (Fig. 2a). The E values were estimated to be 180, 180, 280, and 320 kJ/mol for glycerols with 2, 5, 7.5, and 10 wt% water contents, respectively. The E values were almost the same as those for the 1-alcohols with 6–8 carbons, but were lower than the values for 1-butanol and 1-pentanol (500–700 kJ/mol) [7].

The σ values were 1–10 kJ/mol and hardly depended on the temperature (Fig. 2b). These values were not far from the previously reported values (7–30 kJ/mol) [7]. This fact indicates that the σ value reflects the intrinsic property of the immobilized CALB molecules, but does not depend on the solvent in which the immobilized-enzyme particles are dispersed.

Thermal inactivation of the immobilized CALB at 90 °C was also examined in glycerols with higher water contents. The residual activity in glycerol with a 90 wt% water content after a 1-day incubation was higher than 60% although it was only 5% in distilled water, indicating that the addition of a small amount of glycerol significantly

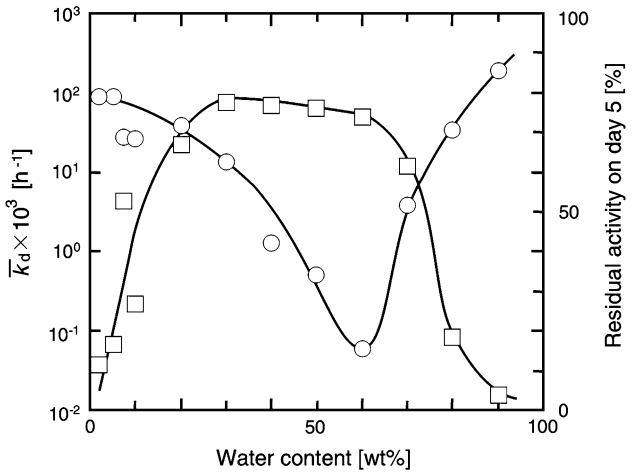

Fig. 3 Dependences of (*open circles*) the rate constant corresponding to the mean free energy of activation, $\bar{k}_d$, and (*open squares*) the residual activities on day 5 for inactivation of the immobilized CALB at 90 °C on the water content of glycerol

stabilized the immobilized CALB. As shown in Fig. 3, the immobilized CALB was extremely stable in glycerols with 30–60 wt% water contents and maintained ca. 75% activity even after a 5-day incubation at 90 °C. The improvement in the stability may be due to the reinforcement of the structure of the lipase molecule by stronger hydrophobic interactions between the nonpolar amino acid residues because it was reported that sugars and polyols strengthen the hydrophobic interactions inside a protein molecule and that the interaction becomes stronger at higher temperature [8].

Meanwhile, the immobilized CALB became unstable in glycerol with a 20 wt% water content or lower. It was also reported that glycerol suppresses the transfer of a hydrophobic group from an aqueous phase to a nonpolar one, and that polyols change the structure of water [8]. Therefore, the decrease in the stability of glycerols with low water contents may be ascribed to imbalances among the stabilization by a hydrophobic interaction, the suppression of transfer of a hydrophobic group, and the change in the structure of water.

Conclusion

The immobilized CALB was stabilized in glycerol containing water, especially in glycerols with 30–60 wt% water contents. The thermal inactivation process did not obey the first-order kinetics, but was expressed by a model in which the free energy of activation for the enzyme inactivation was described in the Gaussian distribution. This fact indicated the heterogeneity during the modification of the enzyme molecules due to their immobilization and suggested the utility of the enzyme at extremely high temperatures.

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