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Static and dynamic half-life and lifetime molecular turnover of enzymes

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The static half-life of an enzyme is the half-life of a free enzyme not working without substrate and the dynamic half-life is that of an active enzyme working with plenty amount of substrate. These two half-lives were measured and compared for glucoamylase (GA) and β-galactosidase (BG). The dynamic half-life was much longer than the static half-life by one to three orders of magnitude for both enzymes. For BG, the half-life of the enzyme physically entrapped in a membrane reactor was also measured. In this case also, the half-life of BG in the membrane reactor was much longer than the free enzyme without substrate. These results suggest the large difference in stabilities between the free enzyme and the enzyme–substrate complex. This may be related to the natural enzyme metabolism. According to the difference in half-life, the lifetime molecular turnover (LMT), which is the number of product molecules produced by a single molecule of enzyme until it loses its activity completely, was much higher by one to four orders of magnitude for the active enzyme than the free enzyme. The concept of LMT, proposed here, will be important in bioreactor operations with or without immobilization.

[**Key words:** Half-life of enzyme; Stability of free enzyme; Stability of enzyme–substrate complex; Lifetime molecular turnover of enzyme; Glucoamylase; β-Galactosidase]

Proteins are known to be marginally stable in an aqueous environment with the very small stabilization energy because of the almost complete enthalpy–entropy compensation (1,2). We have thermodynamically analyzed the marginal stability of proteins in various solutions and established the important role of the water activity in protein stabilities in aqueous systems (3–7). The marginal stability of proteins might be prerequisite for the biological system because proteins, including enzymes, are doomed to be metabolized as soon as they finish their mission. In the protein metabolism, the protein-death system such as the ubiquitin-proteasome and the autophagy-lysozyme play important roles (8–10).

The marginal stability of proteins, however, is a major problem in the industrial use of enzymes. Therefore, tremendous research efforts have been devoted to the stability analysis of thermophilic enzymes (11,12), protein engineering (13), and immobilization of enzymes (14–17) for the improvement of enzyme stability.

In the previous paper, we measured and compared thermodynamic, kinetic, and operational stabilities of yeast alcohol dehydrogenase (18) and found out that the active enzyme with an enough amount of substrate is much stable than the free enzyme without substrate, suggesting the large difference in stabilities between the free enzyme and the enzyme–substrate complex.

In the literature, the half-life of native horse liver alcohol dehydrogenase, physically entrapped in a dialysis membrane system, was measured and compared with that of a free enzyme without substrate at 25 °C and the former was reported to be 14.5 times longer than the latter (19). As for the immobilized enzyme, the effect of the coexistence of substrate on the enzyme stability has been explored for penicillin amylase (20) and glucose isomerase (21). In these cases, enzymes were stabilized by immobilization so that minor effects of substrates were reported on the enzyme stability.

In the present paper, the static half-life of an enzyme, not working without substrate, and the dynamic half-life of an active enzyme, working with a plenty amount of substrate, are measured and compared for glucoamylase (GA) and β-galactosidase (BG) at various temperatures. In addition, the lifetime molecular turnover, which is the number of product molecules by a single molecule of enzyme until it loses its activity completely, is also measured and compared.

MATERIALS AND METHODS

Materials Glucoamylase AF6 from *Rhizopus niveus* (GA) was gifted by Amano Enzyme (Nagoya). β-Galactosidase from *Escherichia coli* (BG), 2-nitrophenyl β-D-galactopyranoside (ONPG), and β-lactose were purchased from Sigma–Aldrich (Tokyo). Soluble starch and sodium azide were obtained from Nacalai Tesque (Kyoto). All other reagents used were of reagent grade.

Measurement of static half-life of enzyme without coexistence of substrate GA at 1.67×10^{-6} mol/L was dissolved in 0.04 M acetate buffer (28.0 mL of 0.2 M acetic acid mixed with 22.0 mL of sodium acetate diluted to 250 mL of water), pH 4.5, and heated at the fixed temperatures from 55 °C to 70 °C. From the heated enzyme solution, aliquots were sampled at intervals and immediately ice-cooled. Soluble starch at 10%, as a substrate, was dissolved in the same buffer and gelatinized at 80 °C for 10 min and kept at the same temperature of the heat treatment of enzyme. Then, an aliquot from the ice-cooled sample of the heat-treated GA was added to the soluble starch solution (5 mL) to start the enzyme reaction for 1 min. Then, the solution was boiled to stop the enzyme reaction and the amount of the product (glucose) was measured to determine the enzyme activity.

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The similar procedure was applied to BG at 2.76×10^{-8} mol/L in 0.05 M Tris buffer, pH 7.30. In this case, the enzyme activity was measured with 0.05 mol/L ONPG, as a substrate, by the change in optical absorbance at 420 nm at the same temperature of the heat-treatment of enzyme.

Measurement of dynamic half-life of enzyme with coexistence of substrate GA at 1.67×10^{-7} mol/L was added to start the enzyme reaction in 50 mL of 10% soluble starch in 0.04 M acetate buffer, pH 4.5, at various temperatures. To this solution, 100 mg/L sodium azide was added to suppress the effect of microorganisms. Aliquots were taken at intervals to measure the concentration of product (glucose). From the time course of the increase in the product concentration, the half-life of the enzyme was obtained.

BG at 1.85×10^{-9} mol/L was added to 50 mL of 0.840 mol/L β-lactose solution in 0.05 M Tris-buffer, pH 7.30, with 100 mg/L sodium azide, to start the enzyme reaction at various temperatures. Aliquots were taken at intervals and the concentration of the product (glucose) was analyzed and the half-life was obtained. In this case, the BG activities with ONPG and β-lactose were different and the former was turned to be 4.85 times higher than the latter. This difference was corrected in the calculation of lifetime molecular turnover.

Enzyme stability in membrane reactor with physically entrapped BG In a small scale membrane reactor (inner volume 5.65 mL) equipped with an ultrafiltration membrane with cut-off MW = 5000 (PBCC04310, Millipore, Billerica, MA, USA), BG was entrapped at 6.56×10^{-8} mol/L. The membrane reactor was immersed in a water bath to control temperature and 0.840 mol/L β-lactose solution in 0.05 M Tris-buffer, pH 7.30, with 100 mg/L sodium azide, was fed continuously at a flow rate of 0.1 mL/min by HPLC pump (PU-980, Jasco, Tokyo).

Analytical method In the enzyme reactions of GA and BG, the product of the enzyme reaction was glucose, which was analyzed by using F-kit glucose (JK International, Tokyo). ONPG was analyzed by optical absorbance at 420 nm by spectrophotometer (V-500, Jasco, Tokyo).

RESULTS AND DISCUSSIONS

Static and dynamic half-life of GA Fig. 1 shows the stability of the free GA in the buffer solution without substrate at various temperatures from 55 °C to 70 °C. In this temperature range, GA lost its activity in several minutes. This inactivation process could be described by the first-order kinetics as follows.

$$k = k_0 \exp(-\alpha_0 t) \quad (1)$$

where k is the enzyme activity at time = t, k_0 is the initial enzyme activity, and α_0 is the inactivation rate constant without coexistence of substrate, which is determined from the slope in Fig. 1. From the α_0, the static half-life of free enzyme, $\tau_{1/2,0}$ (= $\ln 2/\alpha_0$) was obtained.

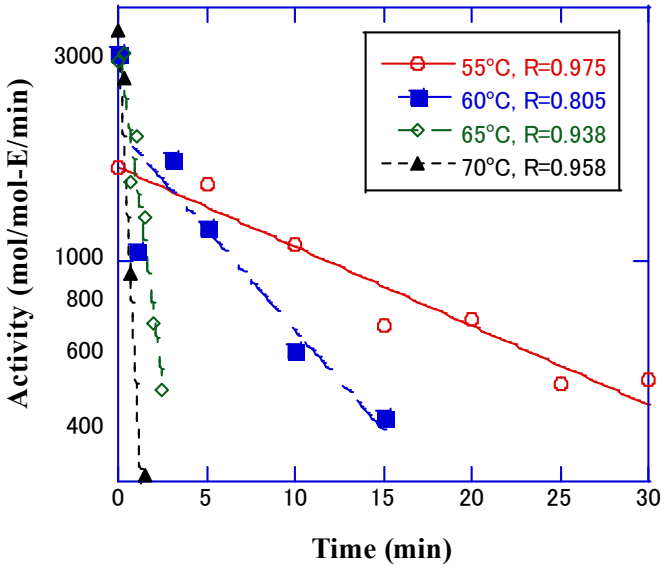

FIG. 1. Static stability of GA in buffer solution without substrate with enzyme concentration at 1.67×10^{-6} mol/L. The enzyme was treated at each temperature for the specified time and the enzyme activity was measured at the same temperature of the thermal treatment.

The dynamic stability of active GA with the coexistence of enough amount of substrate (10% soluble starch) was measured as shown in Fig. 2. In this case, the amount of the product (glucose) was measured from time to time at 60 °C with enzyme concentration varied. From this time course of the increase in the product concentration, it is clear that the enzyme activity continued for several hours, which is much longer than the case in Fig. 1. The apparent saturation curve in Fig. 2 arose from the complete inactivation of the enzyme, not from the completion of the enzyme reaction, because the total substrate concentration was 0.62 mol/L. The time course in Fig. 2 could be described by the integrated form of Eq. 1 as follows.

$$[P]/[E] = \int_0^t k_S dt = (k_{S,0}/\alpha_S)(1 - \exp(-\alpha_S t)) \quad (2)$$

where [P] and [E] are the concentrations of product and enzyme, respectively, k_S is the enzyme activity at time = t, $k_{S,0}$ is the initial activity, and α_S is the inactivation rate constant with the coexistence of substrate. In Fig. 3, the enzyme activity and stability was measured at a fixed enzyme concentration of $[E] = 1.67 \times 10^{-7}$ mol/L with temperature varied. From the curve-fitting to the experimental time-course by Eq. 2, the inactivation rate constant, α_S, was obtained and the dynamic half-life of GA with the coexistence of substrate, $\tau_{1/2,S}$, was calculated.

Fig. 4 compares the static and dynamic half-lives of GA at various temperatures. The latter is much longer by one to two orders of magnitude than the former. These half-lives were determined from the statistically optimized parameters, α_0 and α_S, as shown in Figs. 1 and 3. The error level in α_0 and α_S is mostly less than 20% so that the error in half-lives are also expected at the same level, which cannot explain the large differences in the orders of magnitude between $\tau_{1/2,0}$ and $\tau_{1/2,S}$ in Fig. 4.

Static and dynamic half-life of BG Fig. 5 shows the static stability of the free BG in the buffer solution without substrate at various temperatures from 45 °C to 55 °C. In this temperature range, BG lost its activity in 10–100 min. In Fig. 6, the dynamic BG stability was measured in 0.840 M β-lactose solution at the enzyme concentration of [E] = 1.85×10^{-9} mol/L with

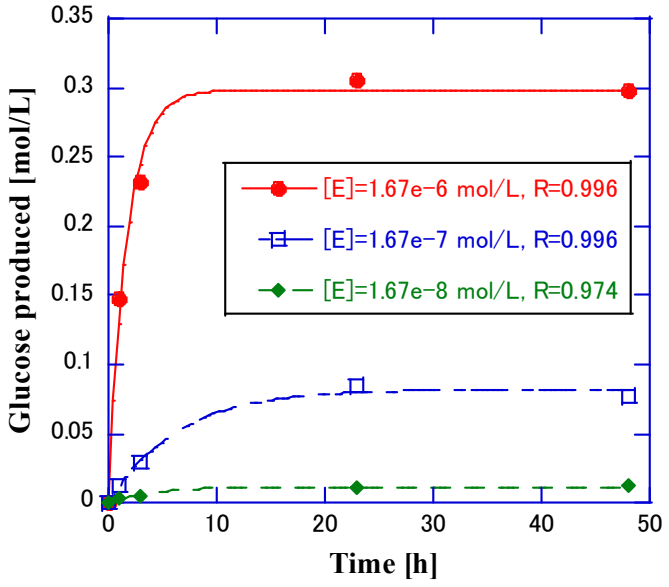

FIG. 2. Dynamic stability of GA in 10% soluble starch at 60 °C with enzyme concentration varied.