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Quantity of Immobilized Acid Phosphatase and Reaction Rate across Enzyme Membranes

Hideo Takeda, Hiroki Tanaka, and Kazuo Nomura*
Department of Chemistry, Faculty of Science, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581

(Received July 7, 2010; CL-100611; E-mail: nomura@chem.rc.kyushu-u.ac.jp)

Acid phosphatase from sweet potato was immobilized on the external-solution side surface of a perfluorocarboxylate ion-exchange membrane. Two membranes of different enzyme amounts were prepared without any crosslinking reagents. The effect of the immobilized-enzyme amount on the enzymatic reaction rate across the membrane was investigated for the hydrolysis of 4-nitrophenyl phosphate. The flux of the reaction product, 4-nitrophenol, was found to obey a Michaelis–Menten equation. As the amount of the immobilized enzyme increased, the Michaelis constant decreased whereas the maximum flux did not increase.

Enzymes exhibit their activity not only in the free form but also in the immobilized state of ionic or covalent-bonding or physical adsorption on an undissolved carrier. While the stability of the enzyme is enhanced by immobilization, the activity of the enzyme is changed compared to that in the free form.[1–5] An immobilized-enzyme membrane reactor is a system in which membrane transport and enzymatic reaction simultaneously take place. The product can be separated, collected, and concentrated based on the difference between the permeability of the membrane to a product and that to the reactants and other products of the enzymatic reaction.

In order to clarify the correlation of the rate of transport of the product (or the reactant) for an enzymatic reaction with the reaction rate, information about the amount of the enzyme immobilized on the membrane is necessary. However, there have been few reports on the quantitative analysis of coupling in membrane reactors based on the determination of the amount of the immobilized enzyme.

In this study, acid phosphatase from sweet potato was immobilized on a perfluorocarboxylate ion-exchange membrane. Hydrolysis of 4-nitrophenyl phosphate (PNPP) across the enzyme membrane was studied. The apparent pK_a of this ion-exchange membrane is 1.9 in a 0.1 mol dm^{-3} NaCl solution.[6] Because the dissociable groups in this ion-exchange membrane is thought to be nearly perfectly ionized in the pH range near the optimum pH of the acid phosphatase, only 4-nitrophenol (PNP) of the hydrolysis product of PNPP permeates through the membrane.[1] Two enzyme membranes with different amounts of the immobilized enzyme were prepared separately. The immobilization procedure in this study involves only adsorption for the enzyme on the membrane surface without any crosslinking reagent. The objective is to determine the quantity of the immobilized enzyme on the membranes and to examine the enzyme–substrate affinity and the enzymatic reaction rate across the enzyme membranes that have different amounts of enzymes.

Acid phosphatase (E.C.3.1.3.2) from sweet potato with a protein concentration of 57 mg mL^{-1} was obtained as a suspension with 1.8 mol dm^{-3} aqueous $(NH_4)_2SO_4$ (Sigma, St. Luis). A perfluorocarboxylate polymer membrane (Flemion 230, kindly supplied by the Asahi Glass Co., Tokyo, with an ion-exchange capacity of 1.4 mmol univalent ion/g dry) was used as the supporting material for the enzyme.

The Michaelis constant for the free enzyme in 0.1 mol dm^{-3} acetate buffer solution (pH 4.8) was first determined. The initial reaction rate was measured at 25 °C by the same method as mentioned in a previous paper.[1] From the Hanes–Woolf plot, the Michaelis constant K_m was determined to be 6.14×10^{-5} mol dm^{-3}. The values of K_m reported in the literature are 6.8×10^{-5} mol dm^{-3} (in 0.2 mol dm^{-3} acetate buffer, 35 °C, pH 5.8)[7] and 1.4×10^{-4} mol dm^{-3} (in 0.1 mol dm^{-3} acetate buffer, 40 °C, pH 5.6).[2]

The dependence of the enzyme activity on the enzyme unit in bulk solution was investigated at a fixed high concentration of PNPP in the saturated region of the initial rate vs. the substrate concentration curve. The initial concentration of PNPP was 5×10^{-4} mol dm^{-3}. A linear relationship exists between the reaction rate and the enzyme unit (correlation coefficient; $R = 0.999$). Using this calibration curve, the change in the amount of the enzyme placed on the membrane after one immobilization was determined.

The perfluorocarboxylate ion-exchange membrane was cut and installed in the cell for the measurement. A mixture of 25 μL of the enzyme solution that was prepared by diluting the stock suspension 10 times by a 1.8 mol dm^{-3} $(NH_4)_2SO_4$ solution with a 25 μL of 0.1 mol dm^{-3} acetate buffer was stirred. Thirty microliters of this mixed suspension was placed on the surface of the external solution side of the membrane. After leaving it at 25 °C for 1 h, the enzyme suspension on the membrane was removed and diluted with 0.1 mol dm^{-3} acetate buffer to analyze the enzyme activity. Two enzyme membranes of different enzyme amounts were prepared. For these enzyme membranes, the immobilization process mentioned above was carried out two or three times. The cell for the measurement with an internal solution of ca. 2 mL of acetate buffer was stored at 5 °C while the flux measurements were not carried out. The amount of the enzyme placed on the membrane for the immobilization, E, and that of the immobilized enzyme on the membrane, E_n, are in Table 1. The immobilized enzyme membrane prepared using the

Table 1. The amount of immobilized enzyme onto the cation-exchange membrane

	E/U[a]	E_1/U[b]	E_2/U[c]	E_3/U[d]	E_{total}/U[e]
M-A	2.13	0.848	0.016	0.021	0.88
M-B	10.04	2.627	0.036	—	2.66

[a]The amount of enzyme placed on the surface of the cation-exchange membrane for immobilization. [b]The enzyme immobilized by the first immobilization. [c]The enzyme immobilized by the second immobilization. [d]The enzyme immobilized by the third immobilization. [e]The sum of E_1, E_2, and E_3.

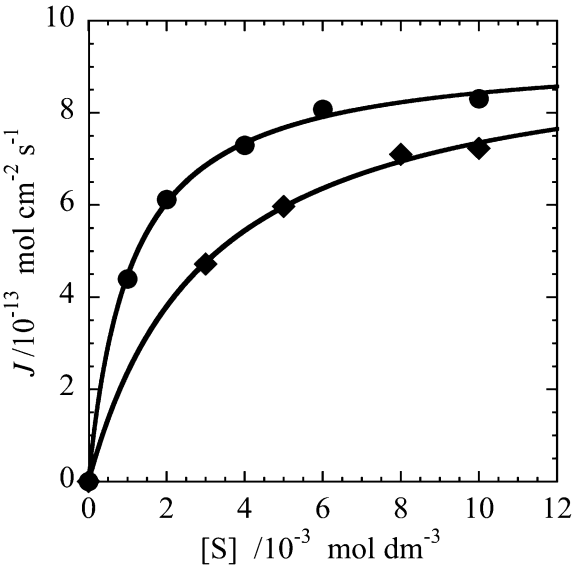

Figure 1. Substrate-concentration dependence of the flux for the immobilized enzyme membrane. (◆) M-A and (●) M-B.

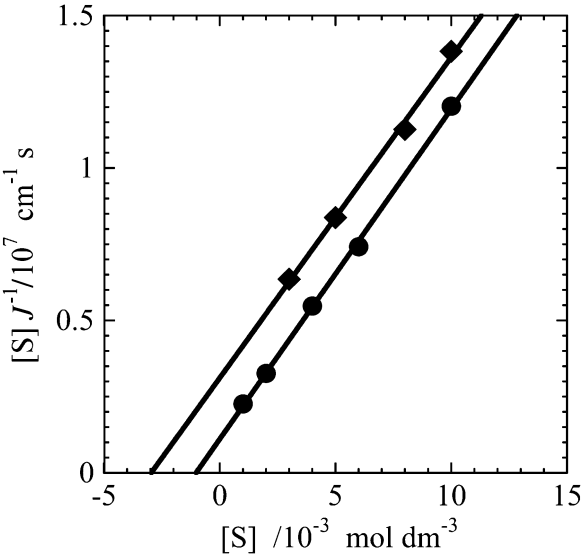

Figure 2. Plot of the Hanes–Woolf type for the immobilized enzyme membrane. The x intercept is $-K_m$ of the immobilized enzyme membrane, and the slope is $1/J_{max}$. (◆) M-A and (●) M-B.

enzyme solution diluted 10 times was designated as M-A, and that using the solution diluted two times as M-B.

Flux measurements were carried out using the same apparatus as described in a previous paper.[1] The layer of the immobilized acid phosphatase was on the external solution side of the membrane. Initially, external and internal solutions contained only 50 and 2 mL of a 0.1 mol dm^{-3} acetate buffer (pH 4.8), respectively. The reaction was started by injecting a small volume of 0.1 mol dm^{-3} PNPP into the external solution. The amount of PNP transported into the internal solution was followed by measuring the time course of the optical absorbance at 317 nm.

In Figure 1, the dependence of the PNP flux into the internal solution in a stationary state on the initial substrate concentration is shown for two enzyme membranes. For both enzyme membranes, the flux shows the Michaelis–Menten behavior versus the initial concentration of the substrate. The results show that the enzymatic reaction on the surface of the enzyme membrane is coupled to the transport of product PNP into the internal solution. Thus, the Michaelis–Menten equation can be applied to the flux of the reaction product so that the flux J is represented by the Michaelis constant K_m, the maximum flux J_{max}, and the substrate concentration [S].[1]

$$J = J_{max}[S]/(K_m + [S]) \quad (1)$$

By rearranging eq 1, we obtain

$$[S]/J = [S]/J_{max} + K_m/J_{max} \quad (2)$$

In Figure 2, the flux divided by the initial substrate concentration is plotted versus the initial substrate concentration (Hanes–Woolf type; eq 2). In this plot, the x intercept and the slope give $-K_m$ and $1/J_{max}$ respectively. The determined values of K_m and J_{max} are shown in Table 2. In both enzyme membranes, the enzyme–substrate affinity was apparently lower than that in the solution. However, the surface of the enzyme layer immobilized on the membrane with a large amount of enzyme was saturated by substrate molecules in the low-concentration range. From these results, the amount of the immobilized enzyme is thought to have an effect on the enzyme–substrate affinity. The value of the maximum flux J_{max} divided by the amount of the immobilized enzyme on the membrane E_{total} can be regarded as the enzyme activity per unit. As the E_{total} increased, the enzyme activity per unit decreased. Compared to the free state of the enzyme, the immobilized enzyme on the membrane would be in a dense state. In the enzymatic reaction, the enzyme forms a complex with the substrate. Thus, the reaction proceeds through a intermediate state formed by the interaction of the substrate with the active site of the enzyme. This suggests a possible effect of the dense state of the enzyme on the formation of the enzyme–substrate complex.

Table 2. Michaelis constant and maximum flux for immobilized enzyme membrane

	K_m /10^{-3} mol dm^{-3}	J_{max} /10^{-13} mol cm^{-2} s^{-1}	$J_{max}E_{total}^{-1}$ /10^{-13} mol cm^{-2} s^{-1} U^{-1}
M-A	3.00	9.99	11.35
M-B	1.00	9.25	3.48

References

1 K. Nomura, M. Liu, *Bull. Chem. Soc. Jpn.* **2006**, *79*, 343.
2 S. Yamato, N. Kawakami, K. Shimada, M. Ono, N. Idei, Y. Itoh, *Anal. Chim. Acta* **2000**, *406*, 191.
3 L. Giorno, E. Drioli, G. Carvoli, A. Cassano, L. Donato, *Biotechnol. Bioeng.* **2001**, *72*, 77.
4 W. Marconi, F. Faiola, A. Piozzi, *J. Mol. Catal., B: Enzym.* **2001**, *15*, 93.
5 T. Uragami, K. Ueguchi, M. Watanabe, T. Miyata, *Catal. Today* **2006**, *118*, 158.
6 Z. Twardowski, H. L. Yeager, B. O'Dell, *J. Electrochem. Soc.* **1982**, *129*, 328.
7 K. Uehara, S. Fujimoto, T. Taniguchi, K. Nakai, *J. Biochem. (Tokyo)* **1974**, *75*, 639.

