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Direct Monitoring of Enzymatic Glucan Hydrolysis on a 27-MHz Quartz-Crystal Microbalance

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Enzyme reactions have been kinetically studied by using a Michaelis–Menten equation (steady-state kinetics), in which the concentration of the enzyme–substrate (ES) complex is hypothesized to be nearly constant during the reaction, because it was difficult to detect the concentration of the ES complex.[1,2] The reaction rate was simply obtained from the initial rate of the product increase. In the case of starch hydrolyses catalyzed by *exo*-glycohydrolases such as glucoamylase, the produced glucose monomers are analyzed by various methods such as the glucose oxidase method,[3,4] colorimetric method,[5,6] and stopped-flow fluorescence.[7] If the formation and decay of the ES complex could be followed during the reaction, more precise or quantitative enzyme kinetics are expected.

In this communication, we describe that the starch-immobilized 27-MHz quartz-crystal microbalance (QCM) is a useful tool to detect directly and quantitatively each step of hydrolysis (the enzyme binding, the hydrolysis, and the enzyme release) from the ES complex formation catalyzed by glucoamylase (see Figure 1). QCMs are known to provide very sensitive mass-measuring devices in aqueous solution, and their resonance frequency is proved to decrease linearly upon the increase of mass on the QCM electrode in a nanogram level.[8,9] Glucoamylase (from *Aspergillus niger,* EC 3.2.1.3, MW: 63 kDa) is known to catalyze the release of β-D-glucose from the nonreducing ends of soluble starch such as amylopectin (α-1,4 glucan with α-1,6 linkages at branch points) and of pullulan (glucan of α-1,6-linked maltotriosyl units).[10] The total steps of (1) the binding of glucoamylase to the glucan substrate (mass increase) and (2) the hydrolysis of substrate (mass decrease) could be observed continuously from time dependences of frequency changes of the QCM (Figure 1B).

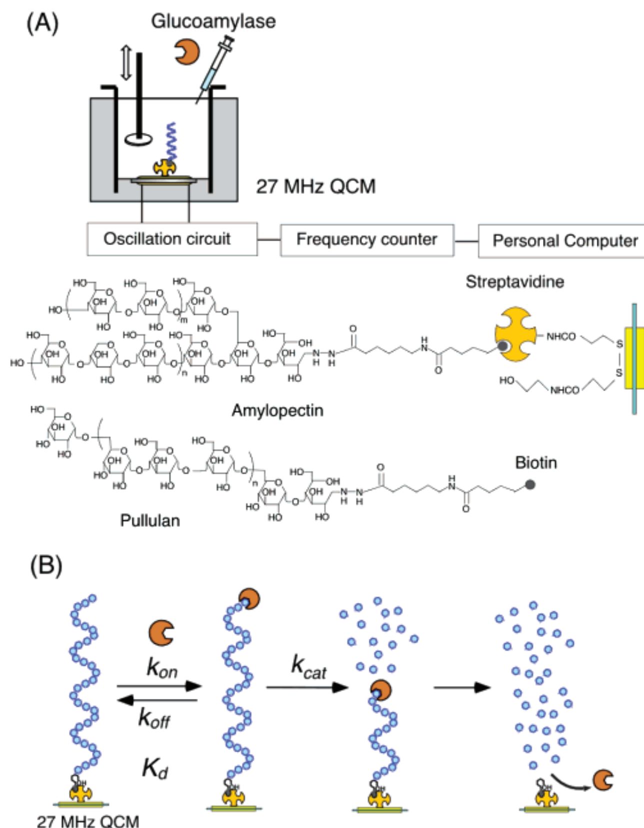

Figure 1. (A) Experimental setup of hydrolysis reactions on the amylopectin- or pullulan-immobilized 27-MHz quartz-crystal microbalance (QCM) in buffer solution and (B) the reaction scheme and kinetic parameters obtained in this work.

An Affinix Q[4] was used as a QCM instrument (Initium Co. Ltd, Tokyo, http://www.initium2000.com) having four 500 μL cells equipped with a 27-MHz QCM plate (8-mm diameter of a quartz plate and an area of 4.9 mm^2 of Au electrode) at the bottom of the cell and the stirring bar with the temperature-controlling system.[9] The 27-MHz QCM was calibrated to change frequency by 1 Hz, responding to the mass increase of 0.62 ng cm^{-2} on the electrode.[9] The biotinylated amylopectin (average MW: 100 $\pm$ 50 kDa) or pullulan (MW: 400 kDa), in which the reducing ends were reacted with biotinamidocaproyl hydrazide, was anchored on a streptavidin-immobilized QCM according to previous papers (Figure 1A).[11] The immobilized amounts of the biotinylated amylopectin or pullulan were maintained to be 150 $\pm$ 10 ng cm^{-2} or 140 $\pm$ 10 ng cm^{-2}, respectively, which correspond to ca. 20% coverage of the surface. This small coverage would give enough space for the binding of a large enzyme molecule.

Figure 2A shows typical frequency changes as a function of time of the amylopectin- or pullulan-immobilized QCM, responding to the addition of different concentrations (16–54 and 540 nM, respectively) of glucoamylase in the aqueous solution. By the addition of enzyme to the glucan-immobilized QCM, the frequency decreased (mass increased) at first due to the binding of glucoamylase to the nonreducing ends of glucans. Then the frequency gradually increased (mass decreased) due to the hydrolysis of substrates on the QCM, and then it reached the constant value ($-$150 $\pm$ 10 ng cm^{-2} over the starting point) independent of the added enzyme concentrations (curves a–d). Since amylopectin or pullulan was immobilized in the amount of 150 $\pm$ 10 or 140 $\pm$ 10 ng cm^{-2} on the QCM plate, this clearly indicates that all of amylopectin or pullulan was hydrolyzed by the enzyme and the enzyme was released from the QCM plate. In the case of the pullulan-immobilized QCM (curve e), the hydrolysis reaction looks very slow even with the high concentration of glucoamylase (540 nM).

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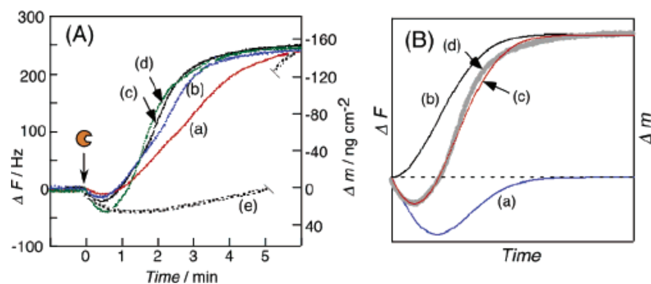

Figure 2. (A) Time courses of frequency changes of the amylopectin-immobilized QCM, responding to the addition of glucoamylase at (a) 16, (b) 27, (c) 38, and (d) 54 nM. The curve (e) shows the hydrolysis of the pullulan-immobilized QCM at 540 nM glucoamylase (25 °C, 20 mM acetate buffer, pH 4.8, 0.1 M NaCl). (B) (a) The theoretical time dependence of [ES] as shown in eq 1, (b) the theoretical time dependence of [P] as shown in eq 2, (c) the fitted curve obtained from simultaneous equations of eqs 1 and 2, and (d) the experimental curve at $[E]_0 = 54$ nM and $[S]_0 = 140$ nm cm^{-2} on a OCM.

Table 1. Kinetic Parameters of Glucoamylase for α-1,4-Glucans

method	substrate	k_{on}/ 10^3 M^{-1} s^{-1}	k_{off}/ 10^{-3} s^{-1}	K_d/ 10^{-6} M	K_m/ 10^{-6} M	k_{cat}/ s^{-1}
QCM[a]	amylopectin	23	0.093	0.0040	–	93
	pullulan	1.8	0.36	0.20	–	2.8
Michaelis–Menten[b]	amylopectin	–	–	–	59	40

[a] Substrate was immobilized on a QCM plate (this work); 25 °C, 20 mM acetate buffer, pH 4.8, 0.1 M NaCl. [b] Glucose oxidase method monitored in micro titer plates and initial rates were analyzed by Michaelis–Menten equation (ref 12); 40 °C, 50 mM acetate buffer, pH 4.5.

In the sigmoidal curves of Figure 2A, time dependences of Δm values reflect both the formation of the ES complex (eq 1) and the hydrolysis of substrate (the product formation, eq 2).

$$[\mathrm{ES}] = [\mathrm{ES}]_{\max}(1 - e^{-t/\tau}) - [\mathrm{P}](1 - e^{-t/\tau}) \quad (1)$$

$$[P] = \frac{k_{cat}}{D_p}\int[\mathrm{ES}]\mathrm{d}t \quad (2)$$

$$\tau^{-1} = k_{on}[E]_0 + k_{off} \quad (3)$$

where, ES, P, and D_p are the enzyme–substrate complex, product, and the degree of polymerization of substrate, respectively. The curves (a) and (b) in Figure 2B were calculated from eqs 1 and 2, respectively. The curves (c) and (d) are the fitted curve obtained from the simultaneous equations both of eqs 1 and 2, and the experimental curve at $[E]_0 = 54$ nM and $[S]_0 = 140$ ng cm^{-2} on the QCM, respectively. The plot of the reciprocal relaxation time versus enzyme concentrations was linear; the slope and y-intercept of the plot yielded values for k_{on} and k_{off}, respectively (eq 3). The dissociation constant (K_d) of enzyme to the substrate was obtained from k_{off}/k_{on}. These kinetic parameters were summarized in Table 1.

The dissociation constant K_d for pullulan was larger than that for amylopectin due to the slow binding (k_{on}) and the fast release (k_{off}) of the enzyme (Table 1). The hydrolysis rate constant in the ES complex (k_{cat}) of pullulan was ca. 30 times slower than that of amylopectin. This reflects the slow reaction of pullulan compared with that of amylopectin (curve (e) of Figure 2A), due to the slow hydrolysis of α-1,6 linkages compared with α-1,4 linkages catalyzed by glucoamylase.[7,10]

When the QCM technique was employed for the starch hydrolysis, all kinetic parameters both of the enzyme binding process (k_{on}, k_{off} and K_d) and the hydrolysis process (k_{cat}) could be obtained simultaneously on the same device as shown in Table 1. In the conventional enzyme reactions in the bulk solution, Michaelis–Menten kinetics have been applied to obtain both the Michaelis constant (K_m) and the hydrolysis rate constant (k_{cat}) according to eq 4. If $k_{off} \gg k_{cat}$, the K_m value is thought to be the apparent dissociation constant ($K_d = k_{off}/k_{on}$).[1,2]

$$K_m = \frac{k_{off} + k_{cat}}{k_{on}} \quad (4)$$

In the Michaelis–Menten kinetics of glucoamylase for amylopectin, $K_m = 5.9 \times 10^{-5}$ M and $k_{cat} = 40$ s^{-1} had been obtained (Table 1).[12] The k_{cat} values obtained from the QCM and the Michaelis–Menten kinetics were relatively consistent with each other; however, the dissociation constants were largely 10^4 times different each other ($K_d = 4.0 \times 10^{-9}$ M from the QCM and $K_m = 5.9 \times 10^{-5}$ M from the Michaelis–Menten kinetics). In the Michaelis–Menten equation, K_m value means the dissociation constant only when $k_{off} \gg k_{cat}$ (eq 4). However, the value of $k_{off} = 9.3 \times 10^{-5}$ s^{-1} was obtained as a very small value compared with $k_{cat} = 93$ s^{-1} from the QCM experiment. Thus, in the case of the α-1,4-glucan hydrolysis such as amylopectin by glucoamylase, K_m value does not reflect the dissociation constant (K_d), and the K_d value obtained from k_{off}/k_{on} by the QCM method reflects the real dissociation constant. Therefore, it is important to grasp all kinetic parameters such as k_{on}, k_{off}, K_d, and k_{cat} on the one device in the enzyme reactions.

In conclusion, the QCM technique will become a new tool to obtain kinetic parameters of various enzymatic reactions, because both the formation of the ES complex and the hydrolysis reaction could be directly observed as mass changes in a nanogram level.

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