Transient kinetic studies of protein hydrolyses by endo- and exo-proteases on a 27 MHz quartz-crystal microbalance

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We have compared endo- and exo-type protease reactions and characterized the enzymatic reaction mechanisms by determining all kinetic parameters (k_{on}, k_{off}, k_{cat}, $K_d = k_{off}/k_{on}$, and $K_m = (k_{off} + k_{cat})/k_{on}$) by following the mass change of the formation and the decay of the enzyme–substrate (ES) complex (k_{on} and k_{off}), and the formation of the product (k_{cat}) on a 27 MHz quartz-crystal microbalance in aqueous solutions. The K_m value was nearly equal to the K_d value for the endo-type protease (subtilisin and α-chymotrypsin); however, in the case of exo-type protease (carboxypeptidase P), the K_m value was quite different from the K_d value, due to $k_{cat} \gg k_{off}$.

Introduction

In general, enzyme reactions have been kinetically studied by using the Michaelis–Menten equation (steady-state kinetics), in which the concentration of the enzyme–substrate (ES) complex has been hypothesized to be nearly constant during the reaction, because of the relative difficulty of measuring the concentration of the ES complex (eqn (1)).[1,2] The reaction rate was simply obtained from the initial rate (v_o) of the product increase by the Michaelis–Menten equation (eqn (2)). From eqn (2), only k_{cat} and K_m values can be obtained, where K_m is a complex value containing k_{cat}, k_{on}, and k_{off}. If the formation and decay of the ES complex could be followed directly during the reaction (transient kinetics), all kinetic values (such as k_{cat}, k_{on}, and k_{off}) could be separately obtained and more detailed enzymatic reaction mechanisms could be discussed.

$$E + S \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} ES \xrightarrow{k_{cat}} E + P \quad (1)$$

$$v_0 = \frac{k_{cat}[E]_0[S]_0}{[S]_0 + K_m} \quad \text{where} \quad K_m = \frac{k_{off} + k_{cat}}{k_{on}} \quad (2)$$

Proteases are popular enzymes, and the hydrolysis kinetics of these enzymes have been studied from the initial rate (v_0) of product increase according to the Michaelis–Menten equation.[3,4] In protease reactions, simple substrates such as di- or oligopeptides have been used in order to avoid effects resulting from the complex tertiary structures of substrates.[5,6] In this paper, we show that all kinetic parameters (k_{cat}, k_{on}, and k_{off}) for both endo- and exo-type protease reactions can be obtained quantitatively by using a protein-immobilized 27 MHz quartz-crystal microbalance (QCM), because the formation and decay of the ES complex, and the formation of the product, can be followed as mass changes on the QCM (Fig. 1).

QCMs are known to provide very sensitive mass-measuring devices in aqueous solutions, and their resonance frequency has been proved to decrease linearly upon the increase of mass on the QCM electrode at a nanogram level.[7,8] Although detection of an ES complex on a plate using QCM and/or atomic force microscopy (AFM) has been performed previously, the results were not analyzed kinetically.[9] Recently, we have reported kinetic analyses for bi-substrate reactions of various enzymes, in which an enzyme binds onto the first substrate on a QCM plate (a preliminary ES complex) and the catalytic rate constant (k_{cat}) can be obtained from the initial rate (v_0) upon the addition of the second substrate.[10] In the case of single-substrate reactions, a curve-fitting method based on deconvolution of the formation of the ES complex and the reduction of substrate on a QCM plate was used.[11] In this study, we applied a curve-fitting method based on a

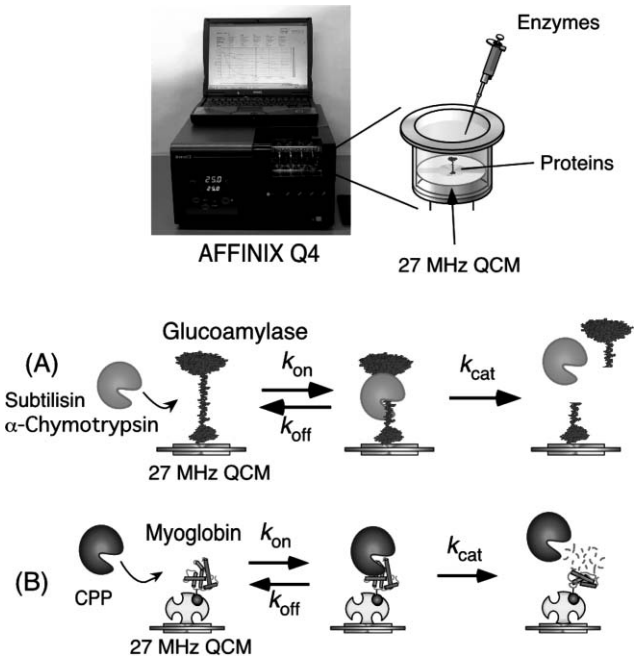

Fig. 1 Schematic illustrations of enzymatic hydrolyses of (A) glucoamylase catalyzed by endo-type subtilisin or α-chymotrypsin and (B) myoglobin catalyzed by exo-type carboxypeptidase P (CPP) on a 27 MHz QCM (AFFINIX Q4). k_{on}, k_{off}, and k_{cat} are the kinetic parameters obtained in this work.

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differential equation related to time for single-substrate reactions in protease hydrolysis reactions as a new and general approach. Thus, we could follow and analyse kinetically the time courses of the formation of the ES complex (k_{on}) as a frequency decrease (a mass increase), the decay of the ES complex (k_{off}) as a frequency increase (a mass decrease), and the formation of the product (k_{cat}) as a frequency increase (a mass decrease).

We chose the site-directed hydrolysis of glucoamylase as a substrate catalyzed by subtilisin (from *Bacillus licheniformis*, EC 3.4.21.62, MW: 27 kDa) or α-chymotrypsin (from *Bos taurus*, EC 3.4.21.1, MW: 25 kDa) as endo-protease reactions. Glucoamylase contains two large domains and a flexible hydrophobic linker, which is a catalytic hydrolysis site for endo-type serine proteases[12] As an exo-type serine protease reaction, we chose myoglobin as a substrate containing only α-helix structures and no S–S linkage, and carboxypeptidase P (CPP from *Penicillium janthinellun*, EC 3.4.16.1, MW: 51 kDa) as an exo-type enzyme that hydrolyzes from the C-terminus of proteins.[13]

Results and discussion

AFFINIX Q4 was used as a QCM instrument (Initium Co. Ltd), having four 0.5 mL cells each equipped with a 27 MHz QCM plate at the bottom, a stirring bar, and a temperature-controlling system (see Fig. 1).[10,11] Calibrations of the 27 MHz QCM in aqueous solutions are described in the Experimental, and 1 Hz of frequency decrease was calibrated to be equivalent to an increase of 0.30 and 0.48 ng cm^{-2} of myoglobin and glucoamylase in aqueous solution, respectively. Glucoamylase (from *Aspergillus niger*) was directly immobilized on the activated carboxyl groups of 3,3′-dithiodipropionic acid activated with *N*-hydroxysuccinimide on the QCM gold surface.[10,11] The immobilized amount was maintained at 210 ± 20 ng cm^{-2} (3.1 ± 0.3 pmol cm^{-2}), indicating almost 65% coverage of the QCM electrode. One amino group of myoglobin (from equine heart) was biotinylated with biotin-$(AC_5)_2$Sulfo-OSu, and anchored on an avidin-immobilized QCM plate according to a previous report.[14] The immobilized amount of biotinylated myoglobin was maintained at 60 ± 5 ng cm^{-2} (3.5 ± 0.3 pmol cm^{-2}), indicating almost 30% surface coverage of the QCM electrode.

Fig. 2 shows typical frequency changes as a function of time of the glucoamylase- or myoglobin-immobilized QCM, responding to the addition of endo-type proteases of subtilisin (2.0 μM), α-chymotrypsin (2.0 μM), or an exo-type protease of CPP (3.2 μM) in aqueous solutions. When the enzymes were added to the protein-immobilized QCMs, frequencies decreased (*i.e.* the masses increased) at first due to the binding of enzymes to protein substrates. Then frequencies gradually increased (*i.e.* the masses decreased) due to hydrolysis of the substrates on the QCMs, and then they reached constant values. Both exo- and endo-type hydrolyses showed similar time courses of frequency changes. In the case of glucoamylase hydrolysis by subtilisin or α-chymotrypsin, *ca.* 100 ng cm^{-2} of substrates was hydrolyzed; almost one half of the immobilized amount (210 ± 20 ng cm^{-2}) (Fig. 2A). This was confirmed by gel-electrophoresis, showing that glucoamylase was hydrolyzed at Val470, near the middle of the linker between 50 kDa and 18 kDa domains.[12] Given the 50% hydrolysis on the QCM plate, it is reasonable to assume complete hydrolysis, since two domains are immobilized with

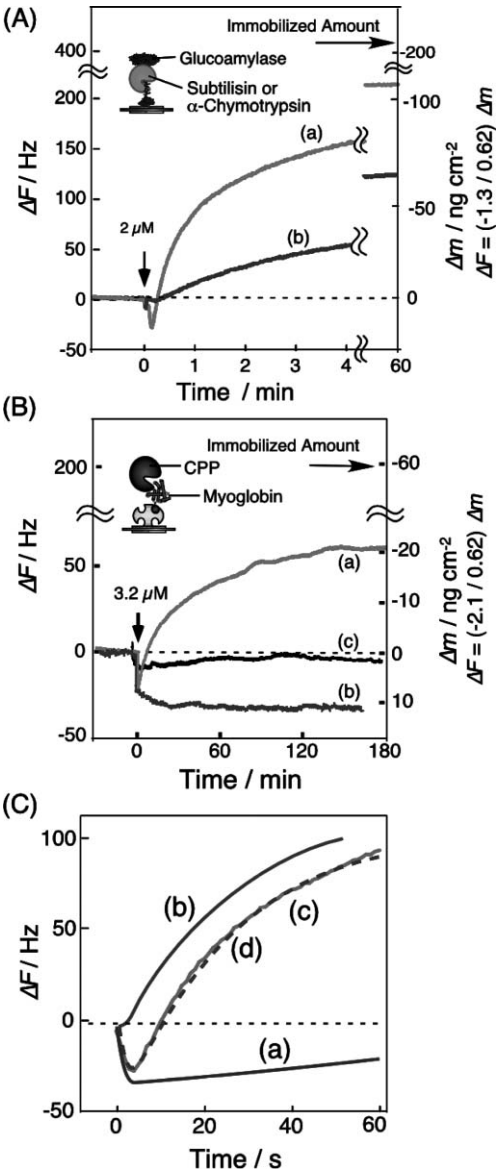


Fig. 2 (A) Typical time courses of frequency changes of the glucoamylase-immobilized QCM, responding to the addition of endo-type proteases of (a) subtilisin and (b) α-chymotrypsin (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM $CaCl_2$ at 25 °C, [glucoamylase] = 210 ng cm^{-2} (3.1 pmol cm^{-2}) on the QCM, [subtilisin] or [α-chymotrypsin] = 2.0 μM in the solution). (B) Time courses of frequency changes of the myoglobin-immobilized QCM *via* biotin–avidin linkage, responding to the addition of exo-type proteases of (a) CPP or (b) CPP inactivated with AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride), and frequency changes of (c) the avidin-immobilized QCM, responding to the addition of CPP (50 mM citrate buffer, pH 3.7, 200 mM NaCl at 25 °C, [myoglobin] = 60 ng cm^{-2} (3.5 pmol cm^{-2}) on the QCM, [CPP] or [inactivated CPP] = 3.2 μM). (C) (a) The theoretical time dependence of [ES] (ΔF_{ES}), (b) the theoretical time dependence of [P] (ΔF_P), and (c) ($\Delta F_{ES} - \Delta F_P$) as a dotted line (eqn 3 in the text), and (d) the experimental curve (a) of Fig. 2A enlarged at the initial part.

random orientations on the QCM. When α-chymotrypsin was injected, a smaller frequency decrease (mass increase) and a slower subsequent frequency increase (mass decrease) were observed than in the case of subtilisin. This suggests that α-chymotrypsin has a

lower substrate binding ability and a slower hydrolysis rate than that of subtilisin.

In the case of the myoglobin hydrolysis by exo-CPP, only 30% of the substrate was hydrolyzed, indicating that the hydrolysis from the C-terminus is not completed even for simple myoglobin containing only α-helices and no S–S linkage (Fig. 2B). When the active Ser-OH group of CPP was inactivated covalently with 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), only the binding process (the initial frequency decrease) was observed and then the frequency did not increase (the hydrolysis did not occur) (curve b in Fig. 2B). When CPP was injected onto the avidin-immobilized QCM, the frequency hardly changed (curve c in Fig. 2B). These results clearly indicate that only active CPP can hydrolyze from the C-terminus of myoglobin containing only α-helices and no S–S linkage, and not avidin having S–S linkages and β-sheet structures, under this reaction conditions.

In the ladle-shaped curve of Fig. 2A, showing a simple one-site hydrolysis of glucoamylase by subtilisin or α-chymotrypsin, the time dependence of ΔF reflects simply the time course of eqn (1), and is shown by the difference between the formation of the ES complex (ΔF_{ES}) and the catalytic hydrolysis (the formation of the product, ΔF_P) (eqn (3)), where MW_E and MW_P are the molecular weights of the enzyme and the released product, respectively. Concentrations of ES and P at the time t are shown in eqn (4) and eqn (5), respectively.

$$\Delta F = \Delta F_{ES} - \Delta F_P = -MW_E[ES] - (-MW_P[P]) \quad (3)$$

$$[ES] = Z(e^{-Xt} - e^{-Yt}) \quad (4)$$

$$[P] = k_{cat} Z \left(\frac{1 - e^{-Xt}}{X} - \frac{1 - e^{-Yt}}{Y} \right) \quad (5)$$

where

$$X = \frac{A - \sqrt{A^2 - 4B}}{2}, \quad Y = \frac{A + \sqrt{A^2 - 4B}}{2}, \quad Z = \frac{k_{on}[E]_0[S]_0}{Y - X},$$

$$A = k_{on}[E]_0 + k_{off} + k_{cat}, \quad B = k_{on}k_{cat}[E]_0$$

The curves (a), (b), and (c) in Fig. 2C are fitted curves of ΔF_{ES}, ΔF_P, and ΔF calculated from eqns (3)–(5), respectively. The experimental curve (d) of the glucoamylase hydrolysis by subtilisin was fitted well by the theoretical curve (c). The kinetic parameters of $k_{on} = (130 \pm 0.9) \times 10^3$ M^{-1} s^{-1}, $k_{off} = 0.20 \pm 0.05$ s^{-1}, and $k_{cat} = 0.08 \pm 0.007$ s^{-1} were obtained by curve-fitting methods, and the results are summarized in Table 1, together with the calculated $K_d = k_{off}/k_{on}$ and $K_m = (k_{off} + k_{cat})/k_{on}$ (from eqn 2) values. When the amount of immobilized glucoamylase substrate on the QCM was changed in the range 110–300 ng cm^{-2} and the concentration of subtilisin was changed from 1.0 to 4.0 μM, none of the kinetic parameters (k_{on}, k_{off}, and k_{cat}) changed within ±10% experimental error. Kinetic parameters for the glucoamylase hydrolysis by α-chymotrypsin (1.0–4.0 μM) were obtained by a similar manner, and are summarized in Table 1.

In the case of multiple hydrolyses from the C-terminus of myoglobin by CPP, the time dependence of the hydrolysis is shown by eqns (3)–(5), in a manner similar to that for the endo-hydrolysis of one site. The theoretical curve was consistent with the experimental curve with the same success to Fig. 2C. The obtained k_{cat}' value shows the single step of the hydrolysis and the real k_{cat} value of multiple attacks by the exo-type enzyme is given by $k_{cat} = nk_{cat}'$, where n is a turnover number of enzymes for one binding. The turnover number was calculated to be $n =$ 51 from 30% of the hydrolysis amount (20 ng cm^{-2}) relative to the immobilized amount (60 ng cm^{-2}) and the number of amino acids in myoglobin (156 aa) (153 × (20/60)). The obtained kinetic parameters are summarized in Table 1. When the amount of immobilized myoglobin substrate on the QCM was varied over the range 20–120 ng cm^{-2} and the concentration of CPP was changed from 1.0 to 4.0 μM, none of the kinetic parameters (k_{on}, k_{off}, and k_{cat}) changed within ±10% experimental error. The myoglobin hydrolysis was also carried out in the bulk solution by changing substrate concentrations ([myoglobin] = 25–100 μM, [CPP] = 1 μM, 50 mM citrate buffer, pH 3.7, 200 mM NaCl, at 25 °C), in which the produced amino acids were followed by absorptions at 570 nm using conventional ninhydrin reactions.[13] The k_{cat} and K_m values were obtained according to the Michaelis–Menten equation (eqn (2)), and the results are summarized in Table 1.

In the endo-type hydrolysis of glucoamylase, subtilisin showed 10 times larger apparent activity ($k_{cat}/K_m = 36 \times 10^3$ M^{-1} s^{-1}) than α-chymotrypsin ($k_{cat}/K_m = 3.1 \times 10^3$ M^{-1} s^{-1}) (see Table 1), which is consistent with apparent curves (a) and (b) in Fig. 2A. This is explained by subtilisin having a large k_{on} value (130×10^3 M^{-1} s^{-1}) compared to that of α-chymotrypsin (19×10^3 M^{-1} s^{-1}), although the other kinetic parameters (k_{off} and k_{cat}) were almost the same. In the exo-type hydrolysis of myoglobin by carboxypeptidase P (CPP) in the bulk solution, only k_{cat} and K_m values could be obtained, and these values were relatively consistent with the k_{cat} and K_m values (calculated from $(k_{off} + k_{cat})/k_{on}$) obtained by the QCM method.

Table 1 Kinetic parameters of the endo-type hydrolysis of glucoamylase by subtilisin or α-chymotrypsin and the exo-type hydrolysis of myoglobin by CPP[a]

Substrates	Enzymes	$k_{on}/10^3$ M^{-1} s^{-1}	k_{off}/s^{-1}	K_d [b]$/10^{-6}$ M	K_m [c]$/10^{-6}$ M	k_{cat}/s^{-1}	(k_{cat}/K_m) [d]$/10^3$ M^{-1} s^{-1}
Glucoamylase	Subtilisin (endo-type)[e]	130	0.2	(1.5)	(2.2)	0.08	(36)
	α-Chymotrypsin (endo-type)[e]	19	0.2	(11)	(13)	0.04	(3.1)
Myoglobin	CPP (exo-type)[f]	10	0.2	(20)	(130)	1.1	(8.5)
	CPP in the bulk solution[g]	—	—	—	(100)	0.3	(3.0)

[a] All kinetic parameters contain ±10% experimental error. Calculated values are shown in parentheses. [b] Calculated from $K_d = k_{off}/k_{on}$. [c] Calculated from $K_m = (k_{off} + k_{cat})/k_{on}$ by eqn 2. [d] Apparent second-order rate constant. [e] 20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM $CaCl_2$ at 25 °C, [glucoamylase] = 210 ng cm^{-2} (3.1 pmol cm^{-2}) on the QCM, [subtilisin] or [α-chymotrypsin] = 1.0–4.0 μM. [f] 50 mM citrate buffer, pH 3.7, 200 mM NaCl at 25 °C, [myoglobin] = 60 ng cm^{-2} (3.5 pmol cm^{-2}) on the QCM, [CPP] = 1.0–4.0 μM. [g] Hydrolysis of myoglobin (25–100 μM) by CPP (0.5 μM) was carried out in aqueous solutions (50 mM citrate buffer, pH 3.7, 200 mM NaCl at 25 °C) by changing the substrate concentration, and k_{cat} and K_m were obtained by the Michaelis–Menten equation (eqn 2).

In Michaelis–Menten kinetics, the K_m value describes the dissociation constant only when $k_{off} \gg k_{cat}$ (eqn (2)). In the endo-type hydrolysis of glucoamylase by subtilisin, the value of $k_{off} = 0.2\ s^{-1}$ was larger than $k_{cat} = 0.08\ s^{-1}$, and the calculated value of $K_m = 2.2 \times 10^{-6}$ M was relatively close to $K_d = 1.5 \times 10^{-6}$ M (see Table 1). For the α-chymotrypsin catalysis, the value of $K_m = 13 \times 10^{-6}$ M was also close to the value of $K_d = 11 \times 10^{-6}$ M. In the exo-type hydrolysis of myoglobin by CPP, however, the calculated value of $K_m = 130 \times 10^{-6}$ M was quite different from $K_d = 20 \times 10^{-6}$ M. This is due to $k_{cat} = 1.1\ s^{-1} \gg k_{off} = 0.2\ s^{-1}$, and the value of $K_m \approx k_{cat}/k_{on}$ from eqn (2) does not therefore equal $K_d = k_{off}/k_{on}$. Thus, in the case of the exo-type hydrolysis of myoglobin by CPP, the value of $K_m = 100 \times 10^{-6}$ M obtained from Michaelis–Menten kinetics in the bulk solution does not reflect the dissociation constant (K_d), and the K_d value obtained directly 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 one device in enzyme reactions.

Although it is difficult to compare the difference between exo- and endo-proteases due to different reaction conditions (pH 7.4 for endo-type subtilisin and pH 3.7 for exo-type CPP), it is useful to compare their kinetic parameters to each other. The value of $k_{cat} = 1.1\ s^{-1}$ for the exo-type CPP was larger than $k_{cat} = 0.08\ s^{-1}$ for the endo-type subtilisin, and the value of $k_{on} = 130 \times 10^3\ M^{-1}\ s^{-1}$ for the endo-type subtilisin was larger than $k_{on} = 10 \times 10^3\ M^{-1}\ s^{-1}$ for the exo-type CPP, but the values of k_{off} were the same for both enzymes ($0.2\ s^{-1}$) (see Table 1). This suggests that the endo-type enzyme quickly finds the specific hydrolysis site of substrates (large k_{on}) and then slowly hydrolyzes the specific site (small k_{cat} and large k_{off}). In contrast, the exo-type enzyme does not find the terminus of the non-specific substrate quickly (small k_{on}), and should hydrolyze the terminal unit quickly and continuously before the release from the substrate (large k_{cat} and small k_{off}). The larger k_{cat} value of the exo-type protease may be explained by the faster release of monomeric amino acids from the substrate. Thus, if k_{cat}, k_{on}, and k_{off} for enzyme reactions could be obtained, we could determine more precisely the reaction mechanisms of exo- or endo-type enzymes.

Conclusions

In conclusion, the QCM technique will become a new tool to obtain kinetic parameters of proteases, because all steps of the formation and decay of the ES complex and the catalytic hydrolysis reaction can be directly observed as mass changes on a nanogram level. This technique could also be useful for finding effective inhibitors of proteases.

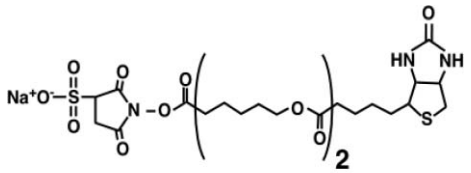
Experimental

Reagents

Subtilisin (from *Bacillus licheniformis*, EC 3.4.21.62, MW: 27 kDa), α-chymotrypsin (from *Bos taurus*, EC 3.4.21.1, MW: 25 kDa), glucoamylase (from *Aspergillus niger*, MW: 68 kDa), and myoglobin (from equine heart, 17 kDa) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Carboxypeptidase P (from *Penicillium janthinellum*, EC 3.4.16.1, MW: 51 kDa) was purchased from TaKaRa Bio Inc. (Shiga, Japan). Biotin-$(AC_5)_2$Sulfo-OSu and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) were purchased from Dojindo Co. (Kumamoto, Japan). *N*-Hydroxysuccinimide (NHS) was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). All other reagents were purchased from Nacalai Tesque Co. (Kyoto, Japan) and used without further purification.

Biotinylation of myoglobin

Myoglobin (60 μM) was treated with biotin-$(AC_5)_2$Sulfo-OSu (30 μM) in 200 μL of a buffer solution (10 mM HEPES, pH 8.0, 200 mM NaCl) for 30 min at room temperature.[14] After addition of Tris buffer (250 mM Tris-HCl, pH 7.4) to quench the reaction, the solution was dialyzed to remove unreacted biotin compounds in a buffer solution (20 mM HEPES, pH 7.4, 200 mM NaCl). It was confirmed by MALDI TOF-MS (AXIMA CFR, Shimadzu, Co. Ltd, Kyoto) that one biotin group had reacted with an amino group of myoglobin (biotinylated myoglobin: $M^+ = 17\,404.4$; native myoglobin: $M^+ = 16\,951.3$).



biotin-$(AC_5)_2$Sulfo-OSu

Immobilization of substrate proteins on the QCM plate

Glucoamylase was covalently immobilized on a 27 MHz QCM plate as follows (amine coupling method).[9] 3,3′-Dithiodipropionic acid was immobilized on a cleaned bare Au electrode, and then carboxylic acids were activated as *N*-hydroxysuccinimidyl esters on the surface. Glucoamylase was treated with activated esters by placing aqueous solutions on the QCM plate.

An avidin-immobilized QCM plate was also prepared by the same amine coupling method. A biotinylated myoglobin was anchored on an avidin-immobilized QCM plate according to a previous report.[14]

QCM setup and calibration in aqueous solutions

AFFINIX Q4 was used as a QCM instrument (Initium Co. Ltd, Tokyo, Japan: http://initium2000.com), having four 0.5 mL cells equipped with a 27 MHz QCM plate (8.7 mm diameter quartz plate, with a 5.7 mm^2 Au electrode) at the bottom of each cell, a stirring bar and a temperature controlling system. Sauerbrey's equation (eqn (6)) was used for the AT-cut shear mode QCM in the air phase,

$$\Delta F_{air} = -\frac{2F_0^2}{A\sqrt{\rho_q \mu_q}}\Delta m \qquad (6)$$

where ΔF_{air} is the measured frequency change in the air phase (in Hz), F_0 the fundamental frequency of the quartz crystal prior to a mass change (27×10^6 Hz), Δm the mass change (in g), A the electrode area (5.7 mm^2), ρ_q the density of quartz (2.65 g cm^{-3}), and μ_q the shear modulus of quartz (2.95×10^{11} dyn cm^{-2}). In the air phase, a 0.62 ng cm^{-2} mass increase per 1 Hz of frequency decrease is expected. However, when QCM is employed for binding

of biomolecules in aqueous solutions, one must consider effects of hydration and/or viscoelasticity of biomolecules (eqn (7)).

$$\Delta F_{water} = -\frac{\Delta F_{water}}{\Delta F_{air}} \frac{2F_0^2}{A\sqrt{\rho_q \mu_q}} \Delta m \quad (7)$$

We therefore directly calibrated the relationship between ΔF_{water} and ΔF_{air} of glucoamylase and myoglobin substrates at different immobilization amounts on a QCM plate. There was a good linear correlation between ΔF_{water} and ΔF_{air} (Δm), with a slope of 1.3 ± 0.2 and 2.1 ± 0.2 for glucoamylase and myoglobin, respectively. Thus, frequency decreases (ΔF_{water}) due to the immobilization of proteins were 1.3–2.1 times larger than those in the air phase (ΔF_{air}), because hydrodynamic water vibrates with proteins. We assumed that the enzyme, bound to the substrate, vibrates with the same hydrodynamic water ratio to the substrate on the QCM plate. Therefore, $\Delta F_{water}/\Delta F_{air}$ values for the combination of glucoamylase and subtilisin (or α-chymotrypsin) or myoglobin and CPP were determined to be 1.3 ± 0.2 or 2.1 ± 0.2, respectively, and the factors of Sauerbrey's equation (eqn (7)) were obtained as $1.3/0.62 = -2.1$ Hz for every increase of 1 ng cm^{-2} (+0.48 ng cm^{-2} for every decrease of 1 Hz) for the glucoamylase hydrolysis, and $2.1/0.62 = -3.4$ Hz for every increase of 1 ng cm^{-2} (+0.30 ng cm^{-2} for every decrease of 1 Hz) for the myoglobin hydrolysis in aqueous solution (see vertical axes of Fig. 2).

The noise level of the 27 MHz QCM was ±1 Hz in buffer solutions at 25 °C, and the standard deviation of the frequency was ±2 Hz over 1 h in buffer solutions at 25 °C. A sensitivity of 0.30–0.48 ng cm^{-2} per −1 Hz is sufficiently large to sense the binding of enzymes.

Enzyme reactions on the QCM plate

A glucoamylase- or myoglobin-immobilized QCM cell was filled with 0.5 mL assay buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM $CaCl_2$ for subtilisin and α-chymotrypsin; 50 mM citrate buffer, pH 3.7, 200 mM NaCl for CPP) until the resonance frequency was held constant (±1 Hz over 30 min) at 25 °C. Frequency changes in response to the addition of the enzyme were followed with time. The solution was vigorously stirred to avoid any effect from the slow diffusion of enzymes. The stirring did not affect the stability and magnitude of frequency changes.

Hydrolysis of myoglobin in the bulk solution

Hydrolyses of myoglobin (25–100 μM) catalyzed by CPP (1 μM) were carried out in 50 mM citrate buffer, pH 3.7, 200 mM NaCl at 25 °C. The amount of the produced amino acid was determined by the colorimetric ninhydrin test at 570 nm.[15]

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