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Precise Evaluation of Enzyme Activity using Isothermal Titration Calorimetry

Nurul Karim and Shun-ichi Kidokoro

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Isothermal titration calorimetry (ITC) has become a powerful method for evaluating enzyme kinetics, as it provides a way to detect catalytic reaction heat as a function of time with high sensitivity and reproducibility. This review focuses on both established and newly emerging methodologies for evaluating the kinetics of enzyme-catalyzed reactions. Because it allows direct determination of the reaction rate, which itself indicates enzyme activity, ITC is expected to provide a general and effective way to evaluate enzyme activity. The theoretical basis of ITC for evaluating enzyme kinetics is reviewed, as demonstrates the advantages of this method.

1. Introduction

Enzymes, the catalysts of biological systems, are remarkable molecular devices that determine the patterns of chemical transformations. For many decades, a great deal of pure and applied studies have been devoted to enzyme reactions, as the mechanisms underlying the special characteristics of the reactions, such as high specificity and high efficiency, have not only attracted scientific interest but also indicated the possibility of employing enzymes as highly useful catalysts in many application fields. Quantitative evaluation of the catalytic activity of enzymes is inevitably important to reveal the enzymes' reaction mechanisms and to use the catalysts most effectively.

To evaluate enzyme kinetics, it is necessary that the transformation of a substrate into a product is accompanied with an observable event. Since the change in enthalpy is one of a reaction's general features, several approaches have been employed to monitor enzyme-catalyzed reactions using calorimetry.[1-17] The heat generated as the reaction proceeds is a direct and sensitive observable quantity. Because it allows the direct determination of the reaction rate, which indicates the enzyme activity itself, calorimetry is expected to provide a general and effective way to evaluate enzyme activity.

ITC provides one possibility for detecting the catalytic reaction heat as a function of time with high sensitivity and reproducibility. Two calorimetric variables, the compensation power and its integral, can be determined directly and precisely by this method.[13-15] Under hydrolytic conditions, the combination of a calorimetric Lineweaver-Burk plot with these two variables and the non-linear least-squares method was found to be effective for determining enzymatic parameters precisely.[13-15] The kinetic parameters, k_{cat} and K_M, obtained from calorimetric observables, clearly indicated that the enzyme-catalyzed hydrolysis reaction is well approximated by a simple Michaelis-Menten equation.[13-15]

Traditionally, ITC analysis has used data obtained only after the enzyme solution has been fully titrated.[13, 14] However, since the enzyme reaction occurs in the cell even during titration, and since the precise total enzyme concentration in the cell is determined by the titration program, it is possible to use the experimental data gathered during titration for the analysis. To determine the reaction heat and enzyme parameters more precisely, the traditional method has been modified to treat all of the hydrolysis data observed by ITC.[15]

In addition to activity measurement, inhibition studies of different enzyme-catalyzed hydrolysis reactions have used ITC with great success.[8-11,14,15] In most models of

enzymatic measurement systems, product inhibition and accumulation are often a problem. In most cases, product inhibition is neglected by measuring the initial reaction rate before product accumulation is obvious. In other cases they are considered in Michaelis-Menten functions, which makes the data analysis more complicated. Thus in all cases product inhibition is regarded as a disadvantage to measurement. However, product inhibition can change the size and shape of the ITC curve, which can be used to dig out other useful information.[6,8] An isothermal titration calorimetric method was developed to evaluate the kinetic parameters of enzyme catalytic reactions by employing the product inhibition as a probe.[7,11]

This review article describes the use of ITC as a universal assay to measure enzyme kinetic parameters. With the expansion of genetic information and the simplification of methods for creating site-directed mutations, the understanding of enzyme functions and mechanisms in comparative enzyme analysis is anticipated. As a precise, rapid, and simple analysis method, ITC can contribute to the complete characterization of enzymes. This review also highlights the enormous potential of ITC as compared to the traditional spectroscopic methods for studying enzyme catalysis.

2. Advantages of calorimetric assay over conventional methods

Traditional assays of enzyme activity use spectrophotometric or chemical detection methods that include 1) continuous assays where rate is determined by continuous measurement of product generation (or substrate depletion), requiring detection of the product or substrate by a spectrophotometer; 2) discontinuous assays where a reaction is stopped at a fixed time points and the product is quantified using mass spectrometry, chromatography, gel electrophoresis, or other analytical methods; and 3) coupled assays where the product of enzymatic catalysis is a substrate for a coupled assay and where the product can be monitored by continuous or discontinuous assay.

With traditional assay methods, however, many enzymes do not have practical assays. It is not always possible or straightforward to measure changes in substrate or product concentration, because i) opaque or turbid solutions interfere with spectrophotometric detection; ii) native, recombinant, and/or mutant enzyme activity is below the assay's detection limit; iii) the substrate or product do not have a chromophore or fluorophore and are too costly or time-consuming to label; iv) discontinuous assays require multiple steps; v) no straightforward coupled reaction exists; vi) coupled assays introduce inaccuracies; vii) substrate/enzyme activity is unknown; and viii) protein function is unknown. Therefore, what is needed is a universal enzyme assay that is precise, rapid, and simple, can be used for activity screening, and is directly applicable to any enzyme-substrate system without the need for chemical tagging. ITC meets these criteria for a universal enzyme assay.

Since heat is the general probe used in calorimetry to monitor the time course of enzymatic reactions quantitatively and continuously, calorimetry can be applied to evaluate the activity of an enzyme for which no direct method is available yet. The advantages of the calorimetric method over the traditional spectroscopic methods have long been discussed.[4-6,13] High-quality kinetic data are obtained using calorimetry, since the rate is determined directly from the thermal power rather than as a derivative of a measured quantity as observed in spectroscopic methods.

A major advantage of using calorimetry to assay enzymes is that the method, differently from optical ones, needs no chromogenic or fluorogenic substrates. A single experiment is enough to generate the kinetic data, and there is no need to "couple" a desired reaction to a second, optically detectable reaction using additional enzymes. Hexokinase, involved in controlling yeast metabolism, is an example of a reaction where neither the substrates glucose and ATP nor the products glucose 6-phosphate and ADP are detectable with a spectrophotometer. The traditional hexokinase assay measures the formation of NADH at 340 nm in the coupled reaction with glucose-6 phosphate dehydrogenase and NAD^+. But the direct ITC measurement of this enzyme's kinetic parameters do not couple enzyme to glucose-6-phosphate dehydrogenase.[12] Moreover, the calorimetric method, which is totally nondestructive and completely general, enables precise analysis of reactions in spectroscopically opaque solutions.[6] The direct and highly precise measurement of the reaction rate by calorimetry has made it possible to apply this method to evaluate the activities of enzymes from different EC classifications, and the simple and complex kinetics and calorimetrically

evaluated kinetic parameters agreed favorably with the published values in most cases.[6)]

3. Data analysis of ITC

Although ITC-evaluated kinetic data are available for various kinds of enzymes, the theoretical basis of using ITC for determining enzyme kinetics has not been described in many papers.[4,6)] In studies of the calorimetric evaluation of protease activity, the hydrolytic ITC data were analyzed most reliably and systematically using certain derivative equations[13)] that were later successfully applied to evaluate the cellulase kinetics.[14)] In those studies, the data were analyzed only after the completion of the enzyme injection into the ITC cell. However, a recent paper[15)] has shown that the analysis can be improved for the complete and precise measurement of enzyme activity by treating all the data, including the compensation power during titration, when the concentration of the enzyme in the cell is increasing.

In ITC, the heat absorbed per unit of time - that is, the power required, $P(t)$, to keep the cell temperature constant - is monitored as a function of time. Two variables of calorimetry, the compensation power, $\delta P(t)$, and its integral, $\delta Q(t)$, are directly related to the reaction rate and substrate concentration, respectively, represented by the following equations:

$$\delta P(t) = V_c \Delta H v(t) \tag{1}$$

$$\delta Q(t) = V_c \Delta H S(t) \tag{2}$$

where v is the reaction rate, S is the substrate concentration, V_c is the cell volume of the calorimeter, and ΔH is the reaction enthalpy accompanying the enzyme reaction. $\delta Q(t)$ can be evaluated from the experimental data, $\delta P(t)$, by numerical integration with Eq.(3).

$$\delta Q(t) = \int_t^\infty \delta P(t) dt \tag{3}$$

Since total enzyme concentration, E_t, changes slowly by the titration of enzyme solution, a new function, $f(t)$, is used. It can be defined as

$$f(t) = E_t(t) / E_f \tag{4}$$

where a constant, E_f, is the final enzyme concentration after the complete injection of the enzyme solution. Corresponding to this extension, the Michaelis-Menten equation for calorimetric observation and its integral form should be changed to

$$\frac{\delta P(t)}{f(t)} = \frac{P_{max}\,\delta Q(t)}{Q_M + \delta Q(t)} \tag{5}$$

$$\frac{1}{P_{max}} [\delta Q_0 - \delta Q(t)] + \frac{Q_M}{P_{max}} \ln \frac{\delta Q_0}{\delta Q(t)} = \int_{t_0}^{t} f(t) dt \tag{6}$$

where the two parameters, P_{max} and Q_M, are defined as

$$P_{max} = V_c \Delta H k_{cat} E_f \tag{7}$$

$$Q_M = V_c \Delta H K_M \tag{8}$$

and the total reaction heat, δQ_0, is defined as

$$\delta Q_0 = \delta Q(t_0) = V_c \Delta H S_0 \tag{9}$$

In this analysis, t_0 is defined as the starting time of the enzyme titration. Therefore, S_0 and δQ_0 correspond to the initial substrate concentration and the total hydrolysis heat of the substrate, respectively.

An extended Lineweaver-Burk equation for calorimetric observation is derived from Eq.(5) as

$$\frac{1}{\delta P(t)^{corr}} = \frac{Q_M}{P_{max}} \frac{1}{\delta Q(t)} + \frac{1}{P_{max}} \tag{10}$$

where the corrected compensation heat, $\delta P(t)^{corr}$, is defined as

$$\delta P(t)^{corr} = \frac{\delta P(t)}{f(t)} \tag{11}$$

Using Eq.(10), two enzymatic parameters, P_{max} and Q_M, were evaluated with the linear plot of the reciprocal of $\delta P(t)^{corr}$ versus that of $\delta Q(t)$. The two fitting parameters were refined by a non-linear least-squares method. With this method, the theoretical function was calculated from Eq.(6), and three fitting parameters, P_{max}, Q_M, and δQ_0, were adjusted to fit the experimental data directly.

A FORTRAN program was custom-written using the SALS non-linear least-squares program.[18)] The modified Marquardt method was used to refine the parameters.

4. Evaluation of the kinetics of enzyme-catalyzed hydrolysis reaction

The results of the successful ITC approaches[14,15)] to evaluate the activity of the enzyme cellulase are discussed. Here, we review mainly the new analysis method, in which ITC has been successfully applied to evaluate the activities of two endoglucanases - an inverting-type enzyme and a retaining-type enzyme -

in the mechanisms of the enzymatic reactions.[15] The inverting-type enzyme was a fungal endoglucanase whose molecular mass was 23 kDa. This enzyme was expressed in yeast and purified. Endoglucanase II (EG II), originating from *Trichoderma reesei*, is a retaining-type endoglucanase and expressed in yeast.[19] The molecular mass of EGII is 56 kDa. Five oligosaccharides - cellobiose (G_2), cellotriose (G_3), cellotetraose (G_4), cellopentaose (G_5), and cellohexaose (G_6) - were purchased from Seikagaku Co. (Tokyo, Japan) and used without further purification. An isothermal titration calorimetry unit of the MCS system (Microcal, MA, USA) was used to conduct all of the ITC experiments. The cell volume of the calorimeter was 1.344 ml. Data were analyzed by the non-linear least-squares method as described in the previous section.

Fig.1 shows a typical time course of the compensation power for a sample cell in the isothermal titration calorimeter. Thermal equilibrium was established at 30.0℃ and pH 4.0, and then the enzyme solution was injected from 122 s to 249 s to start the reaction. The enzyme concentration increased during the titration and reached its final value after completion of the injection. The exothermic heat accompanying the hydrolysis of 1 mM cellohexaose by the endoglucanase was monitored just after the beginning of the enzyme injection (indicated by the arrow in the figure). The start of hydrolysis, at 122 s, is here denoted as t_0. From 122 s, the exothermic power continued to increase till 249 s due to the gradual increase in the enzyme concentration. The reaction proceeded to its maximal rate (V_{max}) at around 249 s with the final enzyme concentration then decayed to the baseline as the substrate was depleted. When all the cellohexaose was hydrolyzed, the observed power returned to almost the pre-injection level under this condition. Although the ITC apparatus used in this study has a time constant of about several seconds, the steepest change of the compensation power during the titration of the enzyme solution was slow enough to be detected by the time constant. If the ITC with large time constant is used, the correction of the time constant (deconvolution) should be done before data analysis.

The new data analysis method[15] was characterized by its ability to evaluate hydrolysis heat during titration, and thus by its ability to evaluate the kinetics of enzyme-catalyzed reactions more precisely by using a more complete

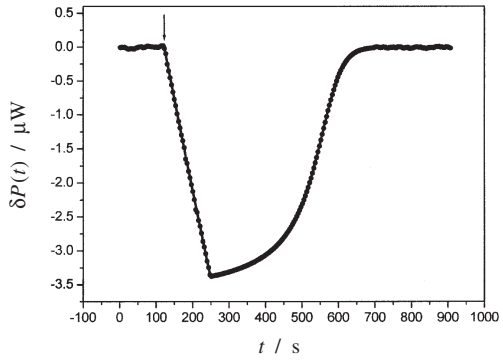

Fig.1 The isothermal titration calorimetric observation of cellohexaose hydrolysis.[15] The inverting-type endoglucanase catalyzed 1 mM cellohexaose hydrolysis at 30℃. The substrate solution was 1.0 mM cellohexaose in 20 mM acetate buffer, pH 4.0, loaded in a 1.344 ml ITC cell, and the enzyme solution in the same buffer was loaded in the syringe. After thermal equilibrium was reached at 30℃, the compensation power was monitored as a function of time with stirring at 41.9 rad s^{-1}. Before injection, the baseline stability was checked. After 122 s, 60 μl of the enzyme solution was injected (indicated by the arrow) for duration of 127 s, giving a final enzyme concentration of 0.27 μM in the ITC cell. The experimental data are plotted as closed circles, and the theoretical curve based on a simple Michaelis-Menten equation is shown as a solid line.

set of data points, including the hydrolysis data during enzyme titration. The non-linear least-squares method refined the rough estimates of P_{max} and Q_M obtained from the Lineweaver-Burk plot of calorimetric observables, as well as that of δQ_0 to fit the experimental hydrolysis data, as shown in the solid line in **Fig.1**. This suggested that the endoglucanase-catalyzed hydrolysis reaction of cellohexaose follows the simple Michaelis-Menten model. From the fitted model, the total hydrolysis heat (δQ_0) was found to be −1138 μJ, and the enthalpy change, ΔH, accompanying the reaction was calculated to be 847 J mol^{-1}. From ΔH and the refined values of P_{max} and Q_M, the k_{cat} and K_M values of the inverting-type endoglucanase to cellohexaose at pH 4.0 can be determined to be 12.1 s^{-1} and 81 μM, respectively, using Eqs.(7) and (8).

A normal-phase HPLC system, consisting of a TSKgel Amide-80 column (Tosoh Co., Tokyo, Japan) in a CTO-6A column oven (Shimadzu, Kyoto, Japan) at 35℃

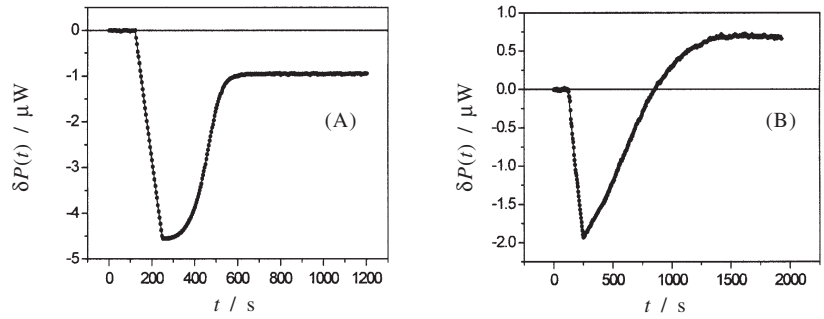

Fig.2 ITC observation of cellohexaose and cellopentaose hydrolysis, catalyzed by the inverting- and retaining-type endoglucanases at 30℃, pH 7.0.[15] (A): 1 mM cellohexaose is hydrolyzed by inverting-type endoglucanase. After thermal equilibrium was reached, 60 μl of enzyme solution was injected at 122 s into the ITC cell for a duration of 127 s. The final enzyme concentration was calculated to be 0.538 μM. The closed circles show the observed power and the theoretical model is represented by the solid line. (B): Hydrolysis of 1 mM cellopentaose was catalyzed by retaining-type endoglucanase. After thermal equilibrium, 60 μl of enzyme solution in a syringe was injected from 122 s to 249 s, giving a final enzyme concentration of 0.214 μM in the ITC cell. Closed circles stand for the observed powers as derived from experimental data and solid lines represent the best-fitted theoretical model.

and an RI8020 refractive index detector (Tosoh), was combined with the ITC approach in order to confirm that the observed heat in **Fig.1** was produced from the hydrolysis of one glycosidic bond. Under these experimental conditions, cellohexaose was completely hydrolyzed to cellobiose and cellotetraose in the ITC cell and the endoglucanase-catalyzed hydrolysis reaction occurring in the ITC cell can be represented by the following scheme.

$$\text{Cellohexaose} + H_2O \rightarrow \text{Cellotetraose} + \text{Cellobiose} \quad (12)$$

In HPLC, the hydrolyzed products were confirmed and their concentrations were evaluated by comparing with the retention times and concentrations of standards (G_2-G_6). The time course of cellohexaose hydrolysis in HPLC was confirmed from the enzymatic parameters obtained from ITC. As observed in the HPLC experiments, the disappearance of cellohexaose as a function of time was in good agreement with the enzyme-catalyzed hydrolysis reaction as observed by ITC.

The inverting-type endoglucanase was found to degrade cellopentaose considerably more slowly than cellohexaose.[14,15] Normal-phase HPLC analysis of the reaction products revealed that cellopentaose was hydrolyzed to cellotriose and cellobiose. The comparison of ITC-analyzed Michaelis constants of this enzyme for cellohexaose and cellopentaose, at the same substrate concentration of 1 mM, suggested that cellopentaose has less affinity for this enzyme than cellohexaose. On the other hand, cellopentaose was found to be preferable to cellohexaose as a substrate for use in ITC observation of the retaining-type endoglucanase (EGII) activity.[15] With a shape almost identical to that in **Fig.1**, the observed ITC data of the retaining-type endoglucanase-catalyzed cellopentaose hydrolysis at pH 4.0 was analyzed clearly and simply by the modified analysis method, with the model considering the hydrolysis of single glycosidic bond based on the simple Michaelis-Menten mechanism.

ITC observation of the cello-oligosaccharide hydrolysis around neutral pH is somewhat different from that observed at pH 4.0. The activities of both the inverting-type and retaining-type endoglucanases were evaluated by ITC at pH 7.0, as shown in **Fig.2**. The main difference in the ITC curves between **Figs.1** and **2** was that, after hydrolysis, the baseline was shifted from the pre-injection level in **Fig.2** but no such shift occurred in **Fig.1**. To determine the possible causes of this discrepancy in the ITC-observed hydrolysis between the two different pHs, it might first be considered that the hydrolysis products of substrates (cellohexaose for the inverting-type and cellopentaose for the retaining-type endoglucanase) might be further hydrolyzed and produce heat. However, this possibility can be denied, since the normal-phase HPLC analysis under the same experimental conditions of ITC confirmed that, in the time range used to analyze ITC

Table 1 Comparative analysis of kinetic parameters of the inverting-type and the retaining-type endoglucanases and expected mutarotation contributions of hydrolysis products at pH 7.0.[15] Because the concentrations of enzymes did not show any significant effect, the evaluated parameters for each enzyme are presented at two different substrate concentrations (0.5 mM, 1.0 mM).

Enzymes	Substrates	Kinetic parameters			P_{mut}^{max}
		k_{cat}	K_M	k_{cat} / K_M	
Inverting	G_6 0.5 mM	7.92 s^{-1}	0.110 mM	72.0 mM^{-1} s^{-1}	−0.440 μW
	G_6 1.0 mM	8.58 s^{-1}	0.130 mM	66.0 mM^{-1} s^{-1}	−0.958 μW
Retaining	G_5 0.5 mM	11.9 s^{-1}	0.250 mM	47.6 mM^{-1} s^{-1}	+0.332 μW
	G_5 1.0 mM	13.7 s^{-1}	0.491 mM	27.8 mM^{-1} s^{-1}	+0.695 μW

P_{mut}^{max}: mutarotation contributions of products.

data, the hydrolysis patterns for both enzymes were the same at pH 7.0 as at 4.0. The probable cause of the shift at pH 7.0 is the mutarotation reaction of hydrolysis products.[15]

In general, when endoglucanase hydrolyzes a glycosidic bond, the newly produced reducing end of the product initially possesses a definite anomer type depending on the hydrolysis mechanism of the enzyme, namely, a retaining-type or an inverting-type. It is well known that the chemical conversion from one anomer to another occurs in water, and that the populations of the two anomers finally reach equilibrium by a phenomenon known as mutarotation

The inverting-type and retaining-type endoglucanases have different catalytic mechanisms: inversion and retention of the substrate's β-glycosidic bond configuration, respectively. The anomeric form of the products formed by an endoglucanase is specific to the enzyme concerned, irrespective of the kind of substrate. The α-anomeric form of products was produced from the substrate cellohexaose by the hydrolysis of inverting-type endoglucanase, and subsequent mutarotation of the products gave equilibrium between the α- and β-anomeric forms. In **Fig.2(A)**, the baseline deviated downward after hydrolysis, indicating the exothermic mutarotation heat produced after hydrolysis of the substrate. Based on the observation of mutarotation heat in ITC, a contrasting result was obtained when cellopentaose was hydrolyzed by the retaining-type endoglucanase at pH 7.0, as shown in **Fig.2(B)**. The hydrolysis products are retained as a β-configuration, and then β to α mutarotation occurs to attain equilibrium. The baseline shifted upward, indicating the endothermic heat from the mutarotation reaction of the hydrolyzed product.

The ITC data analysis method was further extended, and a fitting program was successfully developed to evaluate the kinetic parameters from the hydrolysis reactions by separating the hydrolysis heat from the heat contributed by the mutarotation of products at neutral pHs.[15] The use of only one parameter, P_{mut}^{max} (mutarotation heat contribution of the products), was sufficient for consideration of the mutarotation reaction. Because this is a first-order reaction, the reaction rate is approximated to be proportional to the hydrolyzed product. Moreover, the concentrations of enzymes did not show any effect on the mutarotation contributions of the products.[15]

Table 1 summarizes the kinetic parameters of the two types of endoglucanases as well as the estimated mutarotation contributions of hydrolysis products as evaluated by ITC at pH 7.0. In addition to the kinetic parameters, two remarkable findings on the mutarotation heat were shown in the table. Firstly, the inverting-type endoglucanases produced different signs of mutarotation heat than the retaining type; the former produced exothermic heat while the latter produced endothermic. For both types of enzymes, secondly, it was quantitatively shown that the mutarotation heat depended on the initial substrate concentration. Hence the magnitude of the mutarotation contribution is almost doubled when the substrate concentration is doubled for either type of enzyme.

These results indicate that endoglucanase activity can be calorimetrically evaluated even in cases where the mutarotation is not negligible. Because there is no difference between the initial and final baselines in **Fig.1**, only hydrolysis heat without mutarotation was observed at pH 4.0. From this it can be assumed that the mutarotation rate of the hydrolysis products at pH 4.0 was very slow, and the contribution of heat from this source was expected to be negligible.

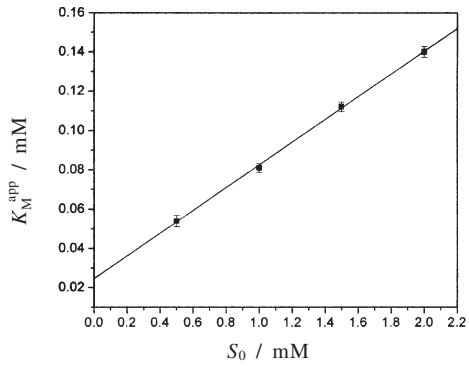

Fig.3 Substrate concentration dependence of K_M values of inverting-type endoglucanase observed by ITC at pH 4.0.[15] Four different concentrations of cellohexaose (0.5 mM, 1.0 mM, 1.5 mM, and 2.0 mM) were used to measure the apparent K_M values of the enzyme. The apparent K_M values versus substrate concentrations were plotted. Using Eq.(13), K_M^0 and K_i values were determined to be 25 ± 1 μM and 424 ± 32 μM, respectively.

Moreover, for the inverting-type endoglucanase, activity depended strongly on the substrate concentration.[14,15] The activity decreased remarkably when the substrate concentration increased. The kinetic parameters of the inverting-type endoglucanase were evaluated by ITC at pH 4.0, using cellohexaose as a substrate at four different concentrations. Although the k_{cat} values remained constant, the increase in the substrate concentration was accompanied by an increase in the apparent K_M values. **Fig.3** shows that the apparent K_M values increased linearly as the initial cellohexaose concentration increased. Using Eq.(13), the apparent competitive inhibition constant, K_i, at pH 4.0 was found to be 424 ± 32 μM.

$$K_M^{app} = K_M^0 \left(1 + \frac{S_0}{K_i} \right) \quad (13)$$

where K_M^{app} is the apparent K_M, K_M^0 is the K_M at the limit of zero substrate concentration, and S_0 is the initial substrate concentration.

X-ray crystallography showed that a cellulase could bind to the substrate and/or products in its substrate-binding sites.[20] This clearly indicates that cello-oligosaccharides become inhibitors as well as substrates. From **Fig.3**, it can be assumed that the substrate and/or the reaction products inhibited the endoglucanase activity competitively. This type of inhibition was initially described as substrate and/or product inhibition[14] since until then it had been uncertain which inhibitor was actually responsible for the dependence of the Michaelis constant on the initial substrate concentration. A series of ITC experiments later confirmed that it was competitive substrate inhibition, because the hydrolysis products, cellotetraose and cellobiose, made very small contributions to the total inhibition, and because the observed inhibition was considered to have occurred due to the substrate.[15]

5. Application of ITC to evaluate the activity of designed mutants

Modern techniques in molecular biology, sequencing, and proteomics have led to a surge in the number of enzymes that can be routinely expressed as recombinant proteins. Site-directed mutagenesis is the most widely used technique to produce highly useful catalysts with altered or enhanced functions. To find out the most plausible outcome of a mutation, however, it is also important to combine the mutational study with an appropriate quantitative analytic method that can evaluate the catalytic activity of the enzyme with high sensitivity and reproducibility. Some recent studies[16,17] clearly indicate that ITC can fulfill the need for a reliable technique offering the capability of measuring accurately the kinetic data to make a comparative analysis among the wild-type and mutant enzymes.

The physical perturbation method has been applied as an effective strategy to design the cellulase mutants, and the activities of all enzymes have been evaluated precisely by ITC.[16] The three-dimensional (3D) structure of the target endoglucanase was estimated using the 3D structure of endoglucanase V (EGV) from *Humicola insolens* by the homology modeling method and molecular dynamic (MD) simulation in water. Basing the numerical calculation of the electrostatic potential on the estimated structure, a single site, Gln138, was designed and selected, where the electric charge introduced by the amino acid replacement was expected to perturb the electrostatic potential around the general base, Asp10, of the enzyme. A negative residue, glutamate, or a positive residue among the lysine and arginine were introduced in the position of Gln138 by site-directed mutagenesis. The wild type and all three mutants - Q138E, Q138R, and Q137K - were expressed in yeast and purified by a published method.[15] The circular dichroism (CD) spectra (J-720, JASCO)

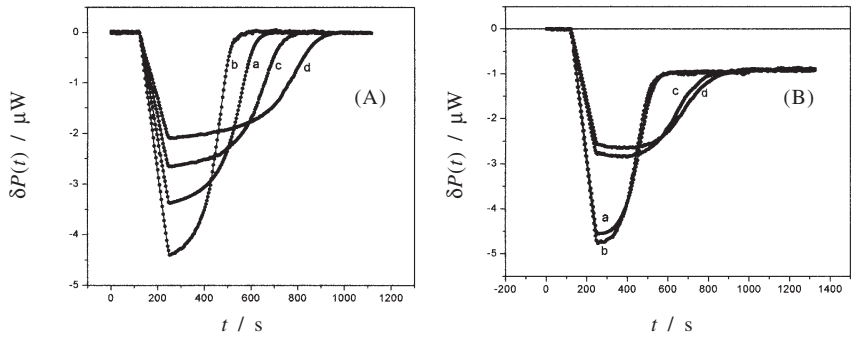

Fig.4 Isothermal titration calorimetric observation of cellohexaose hydrolysis catalyzed by the wild-type and three mutant enzymes at pH 4.0 and 7.0.[16] (A): In four separate experiments with identical ITC conditions, 1.0 mM cellohexaose was hydrolyzed by WT, Q138E, Q138K, and Q138R at pH 4.0. The hydrolysis ITC curves of WT, Q138E, Q138K, and Q138R are represented by a, b, c, and d, respectively. In all the curves, the dotted points show the observed power and solid lines represent the best-fitted theoretical model based on the Michaelis-Menten equation. (B): Hydrolysis of 1 mM cellohexaose catalyzed by four different enzymes at pH 7.0. The final enzyme concentration of ITC cells for all cases was 0.54 μM. The ITC experiments were carried out with the same procedure as described for (A), and a, b, c, and d represent the hydrolysis curves for the enzymes in the same orientation as shown in (A).

and differential scanning calorimetry (DSC) (VP-DSC, Microcal) measurement indicated that the 3D structures of the mutants and their thermal stability were almost the same as those of the wild type.

In ITC, the compensation power is monitored as a function of time. In the previous section it was shown that the heat effect accompanying the mutarotation of the newly produced reducing end of the products couldn't be neglected around the neutral pHs. **Fig.4** shows the ITC observation of the hydrolysis time course of 1 mM cellohexaose catalyzed by wild type and all mutant enzymes at pH 4.0 and 7.0. While the observed heats at pH 4.0 in **Fig.4(A)** are explained by a simple hydrolysis reaction without considering the mutarotation effect, the ITC data in **Fig.4(B)** clearly show the presence of post-hydrolysis at pH 7.0 and are explained very well by the model that considers the heat of the mutarotation reactions of the products. Because the conditions of ITC were identical for all the observations in each figure, the differences in the shapes of the ITC curves of WT (a), Q138E (b), Q138K (c), and Q138R (d) signified the differences in activities among these enzymes. As described earlier for the wild-type enzyme, it was confirmed for the three mutants that the substrate cellohexaose was hydrolyzed to cellobiose and cellotetraose in the ITC cell. Moreover, the kinetic parameters of a single glycosidic bond were evaluated precisely from the solid line of each curve representing the theoretical function of the simple Michaelis-Menten model.

The k_{cat}, K_M, and K_i values for WT, Q138E, Q138K, and Q138R were evaluated precisely across the pH range 3.0 to 8.0. The negative or positive charge at the mutation site increased or decreased the enzyme activity, respectively. Thus in conjugation with ITC, the pH-activity profiles of all individual enzymes were elaborately described, as the pH-dependence showed an electrostatic field perturbation effect on the mutant enzymes.[16] The fundamental finding of that study is that the electric charge introduced by the amino acid replacement at Gln138 can perturb the electrostatic potential of the catalytic base, Asp 10, and thus can perturb the catalytic activity of the enzyme. Furthermore, the positively and negatively charged mutants perturb the catalytic activity in opposite ways.

6. Conclusion

A key focus of biochemistry is determining the activities and functions of enzymes, since every biochemical pathway depends on enzymes for catalysis. Enzymes are also important in drug discovery and development. Approximately half of current drug targets are enzymes, and researchers are discovering new enzymes and developing drugs that interact with them. Notwithstanding

their importance in various contexts of biological life as well as in industry, the present fundamental understanding of how to utilize enzymes is in many respects rudimentary because of certain methodological challenges associated with the study on them. To address these problems, ITC has been successfully applied to quantitatively monitor enzymatic reactions in order to obtain the enzymatic parameters of catalyzed reactions.

The overall picture emerging in this review article is that the application of ITC is a powerful method to evaluate enzyme activity. The strength of this method lies in the universal nature. ITC is becoming a mainstream tool in the study of enzyme kinetics. The capability of a new data analysis method[15] for the complete and precise kinetic characterization of enzymes, along with the subsequent application of this method in a mutational study,[16] has increased the feasibility of using ITC as a general analysis tool for easy and precise measurement of many kinds of enzymes.

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Nurul Karim
Dept. of Bioengineering, Nagaoka Univ. of Technology & Faculty of Pharmaceutical Sciences, Niigata Univ. of Pharmacy and Applied Life Sciences, TEL.&FAX. 025-268-1224, e-mail: nkripon@yahoo.com

Shun-ichi Kidokoro
Dept. of Bioengineering, Nagaoka Univ. of Technology & Genome Science Center, Riken, TEL.&FAX. 0258-47-9425, e-mail: kidokoro@nagaokaut.ac.jp