Netsu Sokutei **28** (2) 74-82

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Calorimetric Evaluation of Protease Activity

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(Received February 2, 2001; Accepted February 20, 2001)

Quantitative evaluation of catalytic activity of enzymes against various substrates under several solution conditions is important to understand the catalytic mechanism. In this paper, the titration calorimetry was applied to evaluate the catalytic activity of a protease not only in the condition of peptide digestion but also in that of peptide synthesis. Two calorimetric observables, the compensation power and its integrals can be determined directly and precisely by titration calorimetry. In the hydrolytic condition, a calorimetric Lineweaver-Burk plot and non-linear least-squares method with these two observables were found to be effective to determine the enzymatic parameters precisely. Moreover, the enthalpy change accompanying the catalytic reaction can be determined with no information on the enzyme concentration and initial substrate concentration by this method with comparing the results of spectroscopic method. In the synthetic condition where the spectroscopic method can not be applied practically, the catalytic activity of the enzyme was shown to be determined quantitatively by titration calorimetry. The feature of direct observation of the reaction rate by calorimetry is considered to give us an effective and precise way to evaluate the protease activity.

1. Introduction

A great deal of pure and applied studies on enzyme reaction have been devoted for many decades because the special characters of the reactions such as high specificity and high efficiency have not only attracted scientific interest on its mechanism but also indicated the possibility to use enzymes as highly useful catalyst in many application fields. Quantitative evaluation of the catalytic activity of enzymes is inevitably important to reveal its reaction mechanism and to use the catalyst most effectively.

In order to design the protein molecule rationally, for example, the quantitative analysis of its catalytic activity is one of the most important process[1] because the information from the evaluation of the mutant protein activity is necessary to improve the designing method in the next step.

The information on the mechanism of the catalysis is also important to design the inhibitors for the enzyme because quantitative analysis of the catalytic reaction against several different substrates will implicate the interaction between the substrates and the enzymes, which is useful for designing the inhibitors for the enzyme.

Spectroscopic method is often used to evaluate the activity because the highly sensitive and continuous measurement can be easily achieved by the method. Lacking the spectroscopic probe in substrates for the enzyme reaction, however, makes it necessary to introduce the probe in the substrate, which might cause the artificial effect on the enzyme reaction. On the other hand, calorimetry has possibility to monitor the catalytic reaction quantitatively and continuously in the many cases where the enthalpy changes accompanying the reaction.

Isothermal titration calorimetry provides one of the possibilities to detect the catalytic reaction heat with the high sensitivity and reproducibility.[2,3] In this paper, a high sensitive isothermal titration calorimeter to monitor the time course of exo- or endo-thermic heat when the enzyme solution was injected to the substrate solution. Moreover the hydrolysis of a substrate was monitored in the same condition as calorimetric

measurement. The comparison between the two different methods provides us the useful parameter for the heat of hydrolysis per a peptide bond at the solution condition precisely, which were necessary to analyze the reaction rate for the condensation reaction.

In this study, thermolysin was used as an example of enzymes to be evaluated by calorimetry. Thermolysin is a thermostable metalloprotease produced by *Bacillus thermoproteolyticus Rokko*[4] and one of the most interesting enzymes both in scientific and practical fields, because of its extremely high thermal stability and relatively narrow specificity.[4,5] The mechanism of catalysis was proposed through the crystallographic studies of thermolysin-inhibitor complexes.[6-10] Recently this enzyme has been used extensively in aspartame (DFM) production: L-aspartyl-L-phenylalanine methyl ester, known as an artificial sweetener that is 200 times sweeter than sucrose. In DFM production, thermolysin plays a key role to catalyze the condensation reaction of an DFM precursor, benzyloxycarbonyl-DFM (Z-DFM), from Z-L-aspartate (Z-Asp) and L-phenylalanine methyl ester (PheOMe).[11,12]

Because the condensation reaction is the key step in DFM production, improvement of this reaction is highly desired. Recently many attempts to improve its catalytic activity by rational designing have been reported and the most improved catalytic activity was realized to be 10 times higher than that of wild type thermolysin.[12-15] In these studies, the catalytic activity for condensation reaction was evaluated by a traditional method to monitor the reaction rate: mixing the enzyme and substrate solution to initiate the reaction, incubating mixed solution, sampling the mixture several times and terminating the reaction for each sample, separating the product from the mixture by chromatography and evaluating the amount of the product. These procedures include time-consuming steps and special techniques are required for precise assay. While the spectroscopic continuous monitoring is an effective way to evaluate the hydrolytic activity of protease, it can not be used to observe the condensation reaction because of high concentration of substrate, required for the condensation reaction.

Direct observables of calorimetry for catalytic reactions relate to the reaction rate, while the traditional method and spectroscopic method monitor the amount of materials as discussed in this paper. In this paper, the condensation reaction was monitored using the special feature of calorimetry.

2. Materials and Method

As the substrate of thermolysin, N-(3-[2-furylacryloyl)-L-aspartate (FA-Asp) and L-phenylalanine amide (Phe-amide) were purchased from Sigma Chem. Co. (USA). Benzyloxycarbonyl-L-Asp (Z-Asp) was from Wako Co. (Japan). They were used without further purification. FA-L-aspartyl-L-phenylalanine amide (FADFA) was synthesized following the previous method.[13] This substrate has been found to show low K_M value for thermolysin and be useful to determine the enzymatic parameters of this enzyme.[13] The substrates were solved in buffer solution and the pH of the solution was adjusted if necessary.

50 mM MOPS buffer including 10 mM $CaCl_2$ and 0.1 μM $ZnSO_4$ pH 6.4 at 37 ℃ was used as buffer solution in this study. The MOPS-Na was purchased from Nakarai Tesch Co. (Japan) and $CaCl_2$ and $ZnSO_4$ were from Wako Co. (Japan). They were used without further purification. pH of buffer solution was adjusted by a pH meter, F-16 (Horiba Co., Japan) with a standard complexed glass electrode No.6366 (Horiba Co., Japan). The pH meter was calibrated with two kinds of standard buffer, pH 4 and 7 from Horiba Co. (Japan). In the pH measurement, the temperature of the solution was kept constant by circulating the water from a thermostat bath circulator, VM-150 (Advantech, Japan).

Thermolysin was purchased from Seikagaku Co. (Japan) as lyophilized powder. The powder was solved nearly 1 mg mL^{-1} with buffer solution. The solvent of the initial solution was exchanged rapidly with ultra filtration using Centriprep-10 (Millipore Co., USA) whose filtered molecular weight is about 10 kDa. The solution after the solvent exchange was filtered with MolCut (Millipore Co., USA) whose filtered molecular weight is 100 kDa in order to remove the fraction of aggregation of the enzymes. The concentration of the enzyme solution was evaluated with a spectrophotometer, UB-35 (Jasco Co., Japan) controlled by a hose-made control software written in F-BASIC on a personal computer, PC98V13 (NEC Co., Japan) with an operating system, Windows 95. The absorbance spectrum was measured from 250 to 350 nm and no absorbance between 320 and 350 nm was checked (below 0.01 OD) in the quartz cell of 1

cm optical path length. The molar extinction coefficient of 60.7 cm^{-1} mM^{-1} was used for the concentration determination. The time-course of absorbance at 345 nm of the substrate solution was also monitored by the spectrophotometer. The temperature of the cell was checked by a digital thermometer CT-500P (Custom Co., Japan) and adjusted by controlled the setting temperature of equipped circulator, EL-15 (Taitec Co., Japan).

An isothermal titration calorimetry unit of MCS system (MicroCal, USA) was used with an attached control software MicroCal Observer ver. 3.0 on a personal computer, Optiplex GX100 (Dell, USA) with an operating system, Windows 95. Typically only one injection of enzyme solution to the substrate solution in the calorimetry cell was used to initiate the enzyme reaction and to monitor continuously the absorbed heat per unit time caused by the hydrolysis. On the other hand, the enzyme solution was injected several times with some time interval to monitor the constant power caused by condensation reaction after each injection. The temperature of the titration cell was set at 37 ℃ and the stirring speed in the calorimeter cell was set to 400 rpm. The cell volume of the calorimeter was 1.344 mL.

According to the Michaelis-Menten equation as shown in the Eq.(1),

$$v = \frac{k_{cat}E_0S}{K_M+S} \tag{1}$$

the reaction rate, v, is described as a function of substrate concentration, S where E_0 is the initial enzyme concentration in the mixture, k_{cat} and K_M are the turnover number and the Michaelis constant, respectively. With the following relation,

$$v(t) = -\frac{dS(t)}{dt} \tag{2}$$

the integral form of Michaelis-Menten equation, Eq.(3) can be obtained,

$$\frac{1}{k_{cat}E_0}[S_0-S(t)]+\frac{K_M}{k_{cat}E_0}\ln\frac{S_0}{S(t)}=t-t_0 \tag{3}$$

where S_0 is the substrate concentration at the time t_0. Although the time t_0 could be set to any time, in this paper t_0 denotes the time when the reaction starts and S_0 is used as the initial substrate concentration.

With isothermal titration calorimetry, the absorbed heat per unit time in calorimeter cell, namely the required power, $P(t)$, to keep the cell temperature constant, is monitored as a function of time. The excess power due to the enzyme reaction can be evaluated as $\delta P(t)=P(t)-P(\infty)$ because the rate of enzyme reaction becomes zero after adequate time.

In the calorimetric observation for time-course of enzyme reaction, the basic relationship between the observable and the rate of the reaction holds:

$$\delta P(t)=V_c v(t)\Delta H \tag{4}$$

where V_c is the cell volume of the calorimeter and ΔH is the enthalpy change accompanying the enzyme reaction. This equation clearly indicates that the direct observation of calorimetry is the reaction rate while that of spectroscopic method is the amount of substrate as shown later.

By integrating both side of Eq.(4) and using Eq.(2), we can obtain the following equation:

$$\delta Q(t)=V_c\Delta H\ [S(t)-S(\infty)] \tag{5}$$

where a new function $\delta Q(t)$ can be evaluated from the experimental data, $\delta P(t)$, by numerical integration with Eq.(6):

$$\delta Q(t)\equiv\int_t^\infty \delta P(t)dt \tag{6}$$

As we can assume $S(\infty)$ is negligible to $S(t)$, we get the following equation from Eq.(5).

$$\delta Q(t)=V_c\Delta H\ S(t) \tag{7}$$

The Eq.(4) and Eq.(7) insist that the reaction rate and the substrate concentration are directly related to the observables of calorimetry.

Using the functions, the Michaelis-Menten equation and its integral form are formulated as the following equations, respectively:

$$\delta P(t)=\frac{P_{max}\delta Q(t)}{Q_M+\delta Q(t)} \tag{8}$$

$$\frac{1}{P_{max}}[\delta Q_0-\delta Q(t)]+\frac{Q_M}{P_{max}}\ln\frac{\delta Q_0}{\delta Q(t)}=t-t_0 \tag{9}$$

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

$$P_{max}=V_c\Delta H\ k_{cat}E_0 \tag{10}$$

$$Q_M=V_c\Delta H\ K_M \tag{11}$$

and the total absorbed heat measured in calorimeter, δQ_0, is defined and has the relationship with the initial substrate concentration in Eq.(12).

$$\delta Q_0 = \delta Q(t_0) = V_c \Delta H \ S_0 \tag{12}$$

The Eq.(8) suggests that the two parameters can be determined from the two observables of calorimetry data. For example, Eq.(13) is derived from Eq.(8), which corresponds to the Lineweaver-Burk equation in traditional way to determine the enzymatic parameters with the reaction rate and the substrate concentration.

$$\frac{1}{\delta P(t)} = \frac{Q_M}{P_{max}} \frac{1}{\delta Q(t)} + \frac{1}{P_{max}} \tag{13}$$

From this equation, the two enzymatic parameters, P_{max} and Q_M can be evaluated with the linear plot of the reciprocal of $\delta P(t)$ versus that of $\delta Q(t)$.

The two parameters can be refined by non-linear least-squares method. The theoretical function, $\delta Q(t)$ is numerically obtained by solving the Eq.(9) at each given time, t, with the given values of t_0 and the fitting parameters, P_{max}, Q_M and δQ_0. With the function, the theoretical power can be calculated from Eq.(8), and the three fitting parameters will be adjusted to fit the experimental data directly. Non-linear least-squares method requires good initial parameters for fitting. The initial values for P_{max}, Q_M are set to the values obtained from the plot of Eq.(13). That for δQ_0 can be set from the observalbe, $\delta Q(t)$ around $t = t_0$.

The house-made FORTRAN program was used with non-linear least-squares package, SALS.[16] The modified Marquardt method was used and can be converged within a few seconds on personal computers.

The hydrolytic reaction of FADFA by thermolysin was monitored also by spectroscopic method. Absorbance spectrum of furylacryloyl acid changes when the peptide bond between Asp and Phe is hydrolyzed. The spectrum change was monitored and analyzed as previously reported.[11] Simply, the absorbance of 2 mL 0.6 mM FADFA in buffer solution at 345 nm was recorded before and after the 40 μL of the enzyme in buffer solution was injected into the substrate solution. It was monitored until the reaction reached to the equilibrium and the absorbance did not change. The change of extinction coefficient, $\Delta\varepsilon_{345}$, accompanying the hydrolysis was 0.661 cm^{-1} mM^{-1}.[13]

With the traditional Lineweaver-Burk plot, the estimates for k_{cat} and K_M were obtained. In detail, two parameters, $V_{max} = k_{cat} E_0$ and K_M are estimated from the plot and the enzymatic parameters can be calculated with the given enzyme concentration. The parameters can be refined by non-linear least-squares method with the integral form of Michaelis-Menten equation, Eq.(3), where the substrate concentrations, $S(t)$, in this equation is evaluated from the absorbance, $A(t)$, as in the following equations.

$$S(t) = \frac{A(t) - A(\infty)}{\Delta\varepsilon_{345}} \tag{14}$$

3. Results and Discussion

A typical time-course of the compensation power for the sample cell of the isothermal titration calorimeter was given in **Fig.1**. After the enzyme solution injection at 4900 s, the exothermic heat accompanying the hydrolysis of the substrate FADFA by thermolysin was observed. The intensity of the compensation power kept almost constant or decreased gradually for a while and changed rather rapidly after 5000 s reaction. When the reaction reached to the equilibrium, the observed power returned to almost the same level as that before the enzyme injection.

The concentration and volume in the experiment in **Fig.1** resembled the reaction condition used for spectroscopic observation with absorbance,[13,14] which indicates the utility and enough sensitivity of calorimetry to observe the hydrolytic reaction of enzymes. The time-course of enzyme reaction from just after the enzyme injection to the end point of the reaction can be observed with enough sensitivity and stability by titration calorimeter. The power due to the enzyme reaction after enzyme injection was calculated easily from the data as $\delta P(t) = P(t) - P(\infty)$. In this case, the power was negative, which apparently shows that ΔH of the hydrolysis of a peptide bond is negative in this solution condition.

In detail, the recorded level of the observed power before enzyme injection was slightly lower than that of end point in **Fig.1**. This was observed in almost all the cases studied and indicated that the enzyme reaction had started at low level before the controlled injection of the enzyme solution. The enzyme solution that could

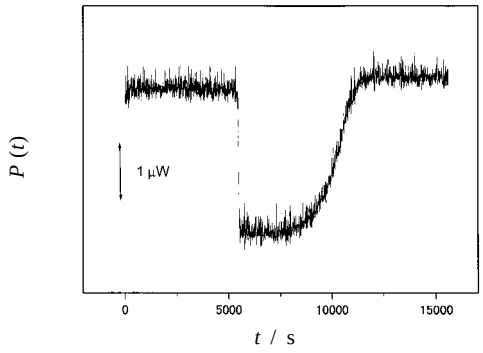

Fig.1 Typical calorimetric observation of dipeptide hydrolysis with thermolysin at 37 ℃. The compensation power of the sample cell was monitored as a function of time with 400 rpm stirring. The substrate solution in the cell was 1 mM Furylacryloyl-Asp-Phe-amide in 50 mM MOPS buffer, 200 mM NaCl, 10 mM $CaCl_2$, 0.1 μM $ZnSO_4$ at pH 6.4. Before injection, the baseline stability was checked. After 4900 s, 40 μL of the enzyme solution was added with 35 s duration. The cell volume of this apparatus, an isothermal titration calorimeter of MCS system (MicroCal, USA) was 1.344 mL. The enzyme concentration of the injected solution was 0.741 μM and that of the calorimeter cell was calculated to be 21.4 nM.

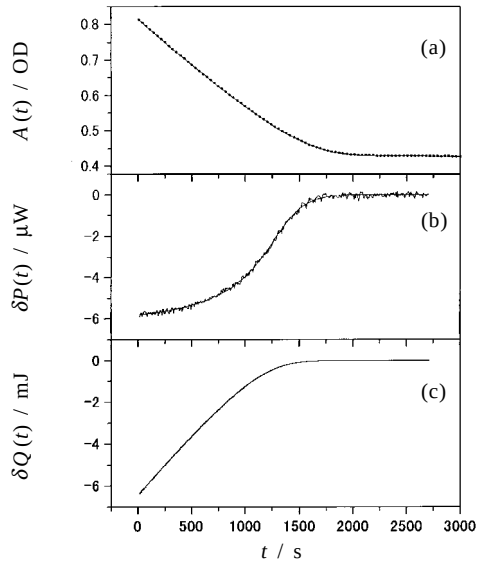

Fig.2 Comparison between the spectroscopic and calorimetric observables in almost the same solution condition at 37 ℃. The substrate solution was 0.6 mM furylacryloyl-Asp-Phe-amide in 50 mM MOPS buffer, 10 mM $CaCl_2$, 0.1 μM $ZnSO_4$ at pH 6.4 and the enzyme solution was 4.89 μM thermolysin in the same buffer solution as substrate. The 40 μL enzyme solution was injected in the 2 mL substrate solution in a standard quartz cell with continuous stirring and monitor the absorbance at 345 nm in a spectrophotometer as shown in (a). The dotted points are the observed time-course of the absorbance and the solid line is the theoretical curve based on a simple Michaelis-Menten model. In the panel (b), the 26.9 μL enzyme solution was injected into the substrate solution of the calorimeter cell and monitor the compensation power for the hydrolytic reaction as a function of time. The plotted points are the observed power and the solid line indicates the theoretical function on a simple Michaelis-Menten model. The observed total heat was calculated from the power as shown in Eq.(6) and plotted in the panel (c). The experimental data were plotted in dotted points and the theoretical curve was in the solid line. They were in so perfect agreement that the difference could not be observed in this panel.

not removed completely around the injection syringe when the enzyme solution was set in the syringe might arise this difficulty. If the outside of the syringe is washed completely after the enzyme solution is filled into it, the amount of leaked enzymes by the washing will not be negligible. On the other hand, the enzyme solution that leaked from the syringe set into the calorimeter cell before the injection might be considered to be the second possibility for the low level enzyme reaction. These artifacts with titration calorimeter could cause the difficulty to determine the enzyme concentration and the initial substrate concentration in the reaction cell as discussed later in detail.

The hydrolytic activity of thermolysin and mutants have been examined with synthetic peptide covalently bound to spectroscopic probe such as furylacryloyl group. The most popular substrate for thermolysin was furylacryloyl-Gly-L-Leu-amide (FAGLA) commercially available.[1,13-15] However its affinity to thermolysin is not enough high to determine the two enzymatic parameters, k_{cat} and K_M, separately but only k_{cat}/K_M could be determined with this substrate. We have found a new substrate FADFA whose K_M is low enough to evaluate the two parameters.[13]

In this reaction, the peptide bond of the synthetic substrate was hydrolyzed automatically under the existence of thermolysin as described in the following scheme:

$$\text{FA-Asp-Phe-amide} + H_2O \rightarrow \text{FA-Asp} + \text{Phe-amide} \quad (15)$$

Because this reaction is reversible, the reverse reaction could be occurred under any condition. The reverse reaction rate, however, was too low to detect it where the product concentration is low. As shown in later, the reverse reaction becomes not only negligible but also the main reaction process when the product concentration increases. Under the low concentration condition around 1mM, a simple Michaelis-Menten mechanism is considered to be the adequately precise model to analyze the time-course of the steady-state enzyme reaction.

The absorbance change at 345 nm of the substrate solution accompanying the hydrolysis was explained completely by the simple Michaelis-Menten equation as shown in the panel (a) of **Fig.2**. With the non-linear least-squares method, the most probable estimates and the estimation error for the two enzymatic parameters, k_{cat} and K_M were $4.61 \pm 0.01\ s^{-1}$ and $69.4 \pm 0.9\ \mu M$, respectively pH 6.4 and 37 ℃ for the hydrolysis of FADFA.

In the panels (b) and (c) in **Fig.2**, the calorimetric observables of the hydrolytic reaction under almost the same condition in the spectroscopic experiment were presented. The exothermic heat was observed and the absolute value of the power due to the reaction decreased in the same manner as seen in **Fig.1** although the enzyme concentration in **Fig.1** was much lower than in **Fig.2**. In the case of exothermic reaction, $\delta P(t)$ and $\delta Q(t)$ are negative as seen in this figure, indicated from Eq.(4) and Eq.(7) with negative ΔH.

The Lineweaver-Burk plot of the calorimetric observables were shown in **Fig.3** using the data in the panels (b) and (c) in **Fgi.2**. A linear relationship between the reciprocals of the two observables clearly indicated that this reaction is well approximated by a simple Michaelis-Menten equation. The rough estimates of P_{max} and Q_M from the linear line fitted to the data were $-6.43\ \mu W$ and $-787\ \mu J$, respectively.

From these values and -6.34 mJ for δQ_0 from the data in the panel (c) in **Fig.2**, the non-linear least-squares method refined these parameters to fit the experimental

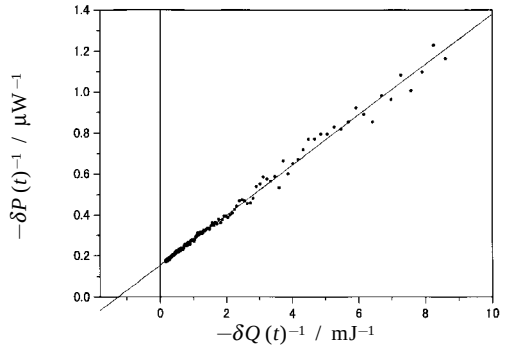

Fig.3 The Lineweaver-Burk plot of calorimetric observables on the hydrolytic reaction of thermolysin with a synthetic substrate FADFA. Two observables, $\delta P(t)$ and $\delta Q(t)$ are the data of Fig.2. The straight line was determined by least-squares fitting.

data, $P(t)$. The solid line in the panel (b) in **Fig.2** represents the fitted function with three adjustable parameters, P_{max}, Q_M and δQ_0, and indicates again that the exothermic reaction is well approximated by this model. The root mean squares residuals of this fitting was 0.09 μW, which agreed with the noise level of this apparatus. The solid line in the panel (c) was calculated from the fitted function and found to agree completely with the experimental data plotted as dotted points.

The most probable values and estimate errors for P_{max}, Q_M and were determined to be $-6.52 \pm 0.02\ \mu W$, -0.819 ± 0.002 mJ and -6.35 ± 0.01 mJ, respectively. The initial values from **Fig.3** were found to be agree roughly with these values. However it was worth while to point out that the values from the Lineweaver-Burk plot could have systematic error, which might occur if the statistical weights of each experimental data were not treated properly. The correct error estimation could be done much easily by non-linear least-squares method. Nevertheless, these data show that these methods are effective to determine the enzymatic parameters.

The K_M values of thermolysin to FADFA was determined spectroscopically, the enthalpy change accompanying the reaction, ΔH can be calculated to be $-8.78 \pm 0.09\ kJ\ mol^{-1}$ using Eq.(11) where the cell volume of the present calorimeter was 1.344 mL. It should be noted that this value can be determined without the information on the concentration of enzyme and substrate. As already discussed, the possibility of the experimental

error on the absolute enzyme concentration, E_0, and the initial substrate concentration, S_0, should be considered in the measurement with titration calorimetry.

From the parameters, k_{cat} and ΔH, the enzyme concentration in calorimeter cell was estimated as 120 nM using the estimated value of P_{max}, which is 1.25 times larger than the calculated value from the volumes of both the cell and injection and the concentration of the injected enzyme solution. At the same time, the initial substrate concentration, S_0, could be estimated from δQ_0 by Eq.(12) as 0.538 mM, which was 0.92 times smaller than the value observed by spectroscopic method. The initial concentration of the present study would not be the true initial one because the leaked small amount of enzyme had already initiated the enzyme reaction in the calorimetry cell. The difference between the apparent initial concentration and that observed in the spectroscopic measurement could be roughly explained by the difference of the enzyme concentration between the observed and calculated values. It indicates further study should be necessary to establish a new method for determining these initial concentrations in titration calorimetry even if the spectroscopic information is not available.

Without the enzymatic parameters observed by spectroscopy, the calculated initial concentration, E_0 and S_0 would be used to calculate the enzymatic parameters and ΔH from calorimetric data. The ΔH might be determined as -8.10 kJ mol^{-1} (its exact value was -8.78 kJ mol^{-1}) from the estimated value for δQ_0. When this value was assigned as ΔH, K_M might be calculated as 75.8 μM (its exact value was 69.4 μM), that for k_{cat} as 6.24 s^{-1} (its exact value was 4.61 s^{-1}), and the value for k_{cat}/K_M as 82.9 s^{-1} mM^{-1} (its exact value was 66.4 s^{-1} mM^{-1}). The errors for ΔH and K_M were derived from the error of S_0, and that for k_{cat}/K_M was from the error of E_0 while that for k_{cat} was from the errors of both S_0 and E_0.

Under the product condition, the reverse reaction of the previous scheme (15) becomes the main process of the catalyzed reaction. Namely the following condensation reaction will be mainly observed when the concentrations of FA-Asp and Phe-amide is higher enough than their equilibrium concentration where the reaction rate of scheme (16) coincides with that of scheme (15):

FA-Asp + Phe-amide
→ FA-Asp-Phe-amide + H_2O (16)

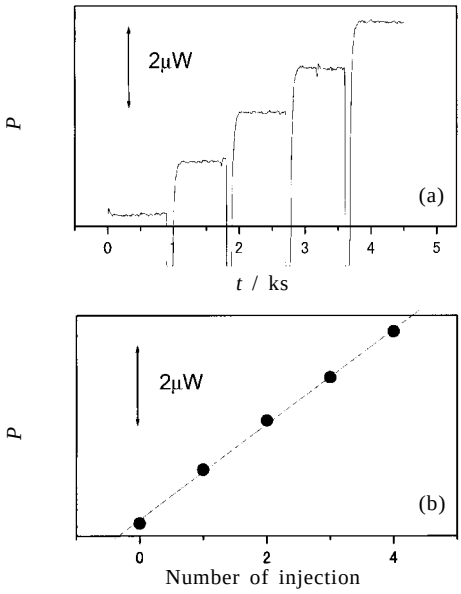

Fig.4 The observed power for the condensation reaction of thermolysin at 37 ℃. The substrate solution of 0.1 M Z-Asp and 0.4 M Phe-amide in 50 mM MOPS buffer, 10 mM $CaCl_2$, 0.1 μM $ZnSO_4$ at pH 6.4. The 20 μL of the enzyme solution (11.5 μM thermolysin) was injected in the substrate solution four times with 900 s interval. The calculated increment of the enzyme concentration by one injection was 169 nM. Other calorimetric measurement condition was the same as Figs.1 and 2. The observed $P(t)$ was plotted as a function of time in panel (a). Although the exothermic reaction due to the dilution heat of substrate observed at each injections were so large to display in this scale, the shift of the power level caused by the enzyme reaction was mainly shown in this panel. The mean value of each constant power level was plotted as filled circles against the injection number. The solid line was determined by linear least-squares method.

Because of the required high concentration of the substrate and the relatively small change of absorbance compared to the intensity, the time-course of the reaction can not be observed spectroscopically.

The time-course of the condensation reaction was observed with calorimeter shown in **Fig.4**. In this reaction, Z-Asp was used instead of FA-Asp in scheme (16). The small amount of enzyme solution was injected four times into the substrate solution. At the injection, large

exothermic heat was observed due to the dilution of the substrate solution, which was indicated from the observation that the same exothermic heat was recorded by the injection of only the buffer solution into the substrate solution (data not shown). After the dilution was completed, the endothermic heat due to the condensation reaction was observed. The reaction rate was considered to be well approximated constant because the concentration of the substrate was approximated to be constant unde the condition of the high substrate concentration. Thus the constant power should be observed as expected in Eq.(4). In the experiment, after about 300 s from each injection, constant power levels were observed. The level increases by almost the same amount in each injection. When only the buffer solution was injected as a negative control, no shift of the power level was observed while the large dilution heat was observed as described above. Because the reaction rate is proportional to the enzyme concentration, the observed power should have the linear relationship with the number of the enzyme injection, which was in fact shown in the panel (b) in **Fig.4**.

The slope of the line in the panel, $1.19 \pm 0.03\ \mu W$, showed the observed power due to the enzyme reaction catalyzed by one injection of the enzyme solution. Using the condensation enthalpy, ΔH, whose absolute value was expected to be the same as that of hydrolysis, 8.78 ± 0.10 kJ mol^{-1}, and the calculated increment of enzyme concentration by one injection, 169 nM, the k_{cat} of the condensation reaction was estimated as 0.60 s^{-1}. at pH 6.4 and 37 ℃.

The k_{cat} value of thermolysin catalyzed condensation reaction at pH 6.0 and 35 ℃ in 0.1 M Tris-maleate buffer for the similar reaction, Z-Asp + PheOMe → Z-Asp-PheOMe was reported to be 0.4 s^{-1},[12] which was determined by a traditional method using quantitative analysis of the amount of the product by reversed phase HPLC. Considering the difference of solution condition and the substrate, the k_{cat} value evaluated by calorimetry in the present study, 0.60 s^{-1}, was considered to agree with the value evaluated by traditional analysis.

In theory the absolute value of the enthalpy change accompanying the condensation reaction should be the same as that accompanying the hydrolysis when the solution condition was identical. In practice, the substrate concentration for condensation reaction will be largely different from that for hydrolysis, and thus it seems to be dangerous to use the value of hydrolysis in the analysis of condensation reaction without any correction. Probably the most effective difference of the solution condition between condensation and hydrolysis would be the ionic strength due to the high concentration of substrates that is ionized in the solution. It should be evaluated by further study how the enthalpy change accompanying the hydrolysis is depend on the ionic strength of the solution by calorimetry using the method presented in this paper.

This study clearly shows that the reaction rate catalyzed by a protease can be precisely and easily obtained by titration calorimetry. Moreover, the normal and reverse reaction can be observed with the same general probe, heat. Its generality will allow us to evaluate almost all kind of enzyme reaction precisely and easily without any other special probe such as chromophore for spectroscopic assay.

Acknowledgment

This work was supported by the Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture.

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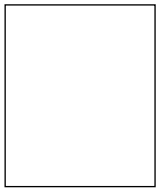
要　旨

様々な溶媒条件下での酵素の触媒活性を定量的に評価することはその触媒機構を理解する上で重要である。この論文では，蛋白質分解酵素について，ペプチドに対する加水分解活性だけでなくペプチド合成に対する触媒活性の評価に滴定型熱量計が応用できることを示した。滴定型熱量計によって，二つの観測量，すなわち熱補償の電力とその積分値が精密に決定できる。加水分解の条件下では，これらの観測量から，いわゆるLineweaver-Burkプロットと非線形最小二乗法によって酵素活性に関するパラメターが精密に決められることがわかった。さらに，加水分解反応に伴うエンタルピー変化を，分光学的な測定の結果と組み合わせることで精度良く求められることを示した。分光学的な測定が不可能である合成反応の条件下では，酵素の触媒活性が定量的に測定できることが明らかになった。熱測定によって直接反応速度が観測できる特質は，酵素活性を評価する上で有効で精度の高い方法を提供すると考えられる。



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