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Calorimetric evaluation of the activity and the mechanism of cellulases for the hydrolysis of cello-oligosaccharides accompanied by the mutarotation reaction of the hydrolyzed products

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Abstract

Isothermal titration calorimetry (ITC) combined with normal-phase HPLC has become one of the most widely applied methods for evaluating the kinetics of cellulase-catalyzed hydrolysis of cello-oligosaccharides, since it allows highly sensitive and precise measurement of the hydrolysis heat of single β-1,4 glycosidic bonds. In this paper, we first extend the previously published analysis of the time course of the enzymatic reaction [N. Karim, S. Kidokoro, Thermochim. Acta 91–96 (2004) 412] in order to evaluate the hydrolysis heat during the enzyme injection, including information of the reaction in the early stage of the enzyme reaction. We then further extend the method in order to evaluate the heat effect accompanying the mutarotation of the newly produced reducing end of the products, which cannot be neglected in neutral pH. By using this method, the activities of two endoglucanases—an inverting-type enzyme and a retaining-type enzyme in the mechanism of the enzymatic reactions—were successfully evaluated against cello-oligosaccharides at pH 4.0 and 7.0. While the observed heats at pH 4.0 were explained by a simple hydrolysis reaction without considering the mutarotation effect, the ITC data at pH 7.0 clearly showed the presence of post-hydrolysis and were explained very well by the new model considering the heat of the mutarotation reaction of the products. The exothermic heat or the endothermic heat caused by the mutarotation reaction was obtained in the case of the inverting- or retaining-type cellulase, respectively. These results indicate that the calorimetric evaluation of the activity of endoglucanases can be done even in cases in which the mutarotation is not negligible, and that the sign of the mutarotation heat can be used to distinguish the anomeric type of the newly produced reducing end of the hydrolyzed products.

Keywords: Isothermal titration calorimetry; Data analysis; Endoglucanases; Normal-phase HPLC; Cello-oligosaccharides; Mutarotation

1. Introduction

Calorimetry is a unique method that enables the direct observation of the evolution or absorption heat caused by the enthalpy change of a chemical reaction. Since the enthalpy change is one of the general features of the reaction, several approaches have been employed to monitor enzyme-catalyzed reactions using calorimetry [1–13]. Because it allows direct determination of the reaction rate, which indicates the enzymatic activity itself, calorimetry is expected to provide a general and effective way to evaluate the enzyme activity.

Isothermal titration calorimetry (ITC) has become a powerful method for evaluating the enzyme kinetics as it provides a way to detect 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 [12,13]. Under the hydrolytic condition, the combination of a calorimetric Lineweaver–Burk plot with these two variables and non-linear least-squares method was found to be

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effective for determining the enzymatic parameters precisely [12,13].

The data used in the analysis of previous studies [12,13] were limited to the part after the completion of titration of the enzyme solution. Because the enzyme reaction occurs in the cell even during the titration, and the precise total enzyme concentration in the cell was determined by the titration program, it is possible to use the experimental data gathered during the titration for the analysis. In order to determine the reaction heat and enzymatic parameters more precisely, we modified the traditional method in order to treat all the hydrolysis data observed by ITC.

To the best of our knowledge, our previous study [13] was the first report to measure the kinetics of an endoglucanase continuously by using ITC. In general, endoglucanases hydrolyze the β-1,4 glycosidic bonds along with the interior of cellulose chains. In addition, enzymatic hydrolysis of the glycosidic bonds is carried out with one of two stereochemical outcomes, net retention or net inversion of the anomeric configuration. Thus the glucosidases are classified into two types, either retaining or inverting. Basic mechanisms for each type were proposed in 1953 [14], and since then there have been numerous studies on the relationship between these mechanisms and the three-dimensional structure of the enzymes [15–19].

The enzyme reported in our previous study was an inverting-type endoglucanase [13]. In this study, a retaining-type endoglucanase was examined, which allowed us to make a comparative study of the activities of the two types of endoglucanases at different pHs.

The choice of the most appropriate substrate is of great importance for the precise and successful measurement of cellulase kinetics. Cello-oligosaccharides were found to be suitable substrates for calorimetric determination of cellulase activity, and the hydrolysis heat of the single glycosidic bond of the substrate can be detected and confirmed by combined use of both the ITC and normal-phase HPLC [13]. The observation of cleavage patterns of cello-oligosaccharides by normal-phase HPLC is necessary to choose the best substrate and the best experimental conditions for evaluating the enzymatic activity of single-bond hydrolysis. The HPLC-determined time course of the degradation of substrates was used to confirm the kinetic parameters by ITC analysis.

Although in our previous study the Michaelis constant was clearly dependent on the concentration of the substrate, it remains uncertain what inhibitor was actually responsible for this dependence [13]. In this paper, we confirmed the individual contributions of the substrate and reaction products to the total inhibition of the enzyme-catalyzed reaction.

Moreover, the hydrolysis products can mutarotate and produce heat [20–22]. In this study, we present a newly extended method that is capable of separating the hydrolysis heat from the contribution made by mutarotation, thereby allowing continuous, simultaneous observation of both the catalysis of cello-oligosaccharides hydrolysis by endoglucanases and the mutarotation heat of the products. The differences in the observed mutarotation heats for the two types of endoglucanases are also discussed.

2. Materials and methods

2.1. A retaining-type enzyme and substrates

Endoglucanase II (EG II) originated from *Trichoderma reesei* was a retaining-type endoglucanase and expressed in yeast as previously published [23]. The molecular mass of EGII is 56 kDa.

Cello-oligosaccharides ($G_{2–6}$) were used as substrates for both the enzymes. Glucose (G_1) was obtained from Nacalai Tesque Inc. (Kyoto, Japan), and five kinds of cello-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. Two buffer solutions: 20 mM acetate buffer (pH 4.0) and 50 mM phosphate buffer (pH 7.0) were used to prepare the substrate and enzyme solutions.

2.2. Preparation of an inverting-type enzyme

The yeast expression vector, pY2831, containing the inverting-type fungal endoglucanase gene was a generous gift of Meiji Seika Kaisha, Ltd., Japan. Construction of the plasmid, pY2831, was reported previously [24,25]. The yeast strain, *Saccharomyces cerevisiae* MS-161 (*MAT*a *trp1 ura3* Suc$^-$), which was used as a host-cell strain for the expression of endoglucanase, was also supplied from Meiji Seika Kaisha, Ltd., Japan. Yeast transformation was carried out by a chemical method using Frozen-EZ Yeast Transformation II^{TM} (Zymo Research, Japan). Transformants were selected in SD-trp medium (Qbiogene, Inc., USA) containing 2% purified agar. For endoglucanase production, transformants were cultivated in SD-trp medium for 48 h, and a part of the culture was inoculated to a final concentration of 1% in SD-trp medium and cultivated for 96–120 h. Culture supernatant was collected by centrifugation, and an equal volume of 20 mM acetate buffer pH 4.0 was added. The final pH of the mixture was adjusted to 4.0 by adding 12N NaOH, and then the mixture was passed through a column (3 cm × 13 cm) containing SP Sepharose^{TM} Fast Flow (Amersham Biosciences, Sweden), a cation exchanger. This column was pre-equilibrated with 20 mM acetate buffer, pH 4.0. The adsorbed enzyme was eluted in the same buffer supplemented with NaCl in a linear gradient from 0 to 0.5 M. The active fractions were pooled and then concentrated and dialyzed by exchanging the buffer, 20 mM acetate buffer, pH 4.0 or 50 mM phosphate buffer for pH 7.0 using the Amicon ultra filtration system (Millipore) with an ultra filtration membrane polyethersulfone having a 10 kDa cut-off. In order to remove the aggregate being produced during dialysis, the enzyme solution was ultra-filtrated with a Molcut ultra filter

unit (USY 20; Advantec, Tokyo, Japan) whose cut-off molecular mass was 200 kDa. The molecular mass of the purified enzyme was determined to be 22,099 Da by a MALDI-TOF mass spectrometer, Voyger-DESTR (Applied Biosystems, Massachusetts, USA), which agreed well with the calculated mass of 22,100 Da based on the amino acid sequence with 7-disulfide-bond formation and the pyroglutamation at the N terminus. Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was done to check the purity. The enzyme concentration was determined by monitoring the absorbance with a UB-35 spectrophotometer (Jasco Co., Japan) using an extinction coefficient of 65.5 cm^{-1} mM^{-1} at 280 nm. The coefficient was determined by evaluating the enzyme concentration using the bicinchoninic acid (BCA) method (Pierce Chemical, USA), with bovine serum albumin (BSA) as the standard of protein concentration.

2.3. Isothermal titration calorimetry

An isothermal titration calorimetry unit of the MCS system (Microcal, Massachusetts, USA) was used with a control-software provided by the manufacturer, Microcal Observer 3.0, on an Optiplex GX 100 PC personal computer (Dell, USA) running Windows 95. The temperature of the titration cell was kept at 30 °C, and the stirring speed in the calorimeter cell was 41.9 rad s^{-1}. The cell volume of the calorimeter was 1.344 ml.

In our previous study [13], we used ITC to evaluate cellulase kinetics by using the derivative equations that had been successfully applied to evaluate protease activity [12]. In those studies of ITC data analysis, only the data after the completion of the enzyme injection into the ITC cell were analyzed. In this paper, we have improved the analysis for complete and precise measurement of enzyme activity by treating all the data, including the compensation power during the titration when the concentration of the enzyme in the cell is increasing.

In ITC, the heat absorbed per unit time—that is, the power required, $P(t)$, to keep the cell temperature constant—is monitored as a function of time. The excess power created by the enzyme reaction was evaluated as $\delta P(t) = P(t) - P(\infty)$ because the rate of enzyme reaction eventually becomes zero. 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 the substrate concentration, V_c the cell volume of the calorimeter, and ΔH 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):

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

In this analysis, the extended Michaelis–Menten equation given below can be used where the total enzyme concentration, E_t, changes slowly by the titration of enzyme solution:

$$v(t) = \frac{k_{cat} E_t(t) S(t)}{K_M + S(t)}. \tag{4}$$

The integral form of the equation is

$$K_M \ln \frac{S_0}{S(t)} + [S_0 - S(t)] = k_{cat} \int_{t_0}^{t} E_t(t)\, dt, \tag{5}$$

where S_0 is the substrate concentration at $t = t_0$. When we define a function, $f(t)$, as

$$f(t) = \frac{E_t(t)}{E_f}, \tag{6}$$

where a constant, E_f, is the final enzyme concentration after the complete injection of the enzyme solution. Eqs. (4) and (5) can be written as

$$\frac{v(t)}{f(t)} = \frac{k_{cat} E_f S(t)}{K_M + S(t)}, \tag{7}$$

$$K_M \ln \frac{S_0}{S(t)} + [S_0 - S(t)] = k_{cat} E_f \int_{t_0}^{t} f(t)\, dt. \tag{8}$$

Corresponding to this extension, the Michaelis–Menten equation for calorimetric observation and its integral form should be changed to

$$\frac{\delta P(t)}{f(t)} = \frac{P_{max} \delta Q(t)}{Q_M + \delta Q(t)}, \tag{9}$$

$$\frac{1}{P_{max}} [\delta Q_0 - \delta Q(t)] + \frac{Q_M}{P_{max}} \ln \frac{\delta Q_0}{\delta Q(t)} = \int_{t_0}^{t} f(t)\, dt, \tag{10}$$

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

$$P_{max} = V_c \Delta H k_{cat} E_f, \tag{11}$$

$$Q_M = V_c \Delta H K_M \tag{12}$$

and the total reaction heat, δQ_0, is defined as

$$\delta Q_0 = \delta Q(t_0) = V_c \Delta H S_0. \tag{13}$$

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. (9) as

$$\frac{1}{\delta P(t)^{corr}} = \frac{Q_M}{P_{max}} \frac{1}{\delta Q(t)} + \frac{1}{P_{max}}, \tag{14}$$

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

$$\delta P(t)^{\mathrm{corr}} = \frac{\delta P(t)}{f(t)} \tag{15}$$

and Eq. (3) becomes

$$\delta Q(t) = \int_t^{\infty} \delta P(t)^{\mathrm{corr}}\, \mathrm{d}t. \tag{16}$$

Using Eq. (14), two enzymatic parameters, $P_{\max}$ and Q_{M}, were evaluated with the linear plot of the reciprocal of $\delta P(t)^{\mathrm{corr}}$ 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. (10), 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 a non-linear least-squares program package, SALS [26]. The modified Marquardt method was used for the parameter refinement.

2.4. Data analysis of ITC with considering mutarotation heat

The analysis method of the previous section was further extended to analyze the hydrolysis data, including the slow but distinct mutarotation heat of the hydrolyzed product. 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. Because this reaction is a first-order reaction, the reaction rate is approximated to be proportional to the hydrolyzed product, $S_0 - S(t)$. Then the mutarotation power, P_{mut}, can be expressed as

$$P_{\mathrm{mut}} = V_{\mathrm{c}} \Delta H_{\mathrm{mut}} k [S_0 - S(t)] \exp[-k'(t - t_0)], \tag{17}$$

where k is the rate constant of the mutarotation of the definite anomer and ΔH_{mut} the molar enthalpy change accompanying the mutarotation, which should be decayed exponentially with an apparent rate constant, k', which should correspond to the sum of the forward and backward rate constants of the mutarotation in theory. Therefore, P_{mut} can be estimated from $\delta Q(t)$, which can be determined by solving Eq. (10) as

$$\begin{aligned} P_{\mathrm{mut}}(t) &= \frac{k \Delta H_{\mathrm{mut}}}{\Delta H} [\delta Q_0 - \delta Q(t)] \exp[-k'(t - t_0)] \\ &= P_{\mathrm{mut}}^{\max} \frac{\delta Q_0 - \delta Q(t)}{\delta Q_0} \exp[-k'(t - t_0)], \end{aligned} \tag{18}$$

where a new constant, $P_{\mathrm{mut}}^{\max}$, which is defined as

$$P_{\mathrm{mut}}^{\max} = k \Delta H_{\mathrm{mut}} S_0 V_{\mathrm{c}} \tag{19}$$

is introduced to express the magnitude of the mutarotation effect. Therefore, the observed power, P_{obs}, is considered to be the sum of those of hydrolysis and mutarotation:

$$\begin{aligned} P_{\mathrm{obs}}(t) &= \delta P(t) + P_{\mathrm{mut}}(t) \\ &= f(t) \frac{P_{\max} \delta Q(t)}{Q_{\mathrm{M}} + \delta Q(t)} + P_{\mathrm{mut}}^{\max} \frac{\delta Q_0 - \delta Q(t)}{\delta Q_0} \\ &\quad \times \exp[-k'(t - t_0)] \end{aligned} \tag{20}$$

A fitting program was developed to optimize the parameters to fit the experimental data with the non-linear least-squares method. In practice, however, the parameter k' was very small under the conditions employed in this study, and it was not determined to be a significant fitting parameter. Therefore, this parameter was fixed at zero in the current analysis and this model can explain all the experimental data. In this study, then, the use of only one parameter, $P_{\mathrm{mut}}^{\max}$, was sufficient for consideration of the mutarotation reaction, as will be shown later.

2.5. Normal-phase HPLC

The normal-phase HPLC system used consisted of a TSKgel Amide-80 column (Tosoh Co., Japan) in a column oven, CTO-6A (Shimadzu, Japan), at 35 °C. An RI8020 refractive index detector (Tosoh Co., Japan) was used to measure the refractive index. Acetonitrile/water (70/30, v/v) was found to be a suitable eluent for proper separation and quick retention of the cello-oligosaccharides in this study. The flow rate was 1.0 ml/min using a CCPM HPLC pump (Tosoh Co., Japan). In all the experiments, samples were taken at different time points, the enzymatic reaction was stopped, and hydrolysis reaction products were analyzed by HPLC as described previously [13]. The retention times of the hydrolysis products were compared to the standards for cello-oligosaccharides.

3. Results

3.1. Evaluation of the kinetics of the inverting-type endoglucanase by ITC at pH 4.0

Fig. 1A shows a typical time course of the compensation power for a sample cell of the isothermal titration calorimeter. Thermal equilibrium was established at 30.0 °C, and then the enzyme solution was injected at from 122 to 249 s for the start of the reaction. The enzyme concentration was increasing during titration, and after completion of the injection, the enzyme concentration reached its final value. Fig. 1B shows the time dependence of the enzyme concentration. The exothermic heat accompanying the hydrolysis of 1 mM cellohexaose by the inverting-type endoglucanase was monitored from the start of the injection of enzyme solution into the cell. The start of hydrolysis, 122 s, is here denoted as t_0. From 122 s, the exothermic power continued to increase till 249 s due to

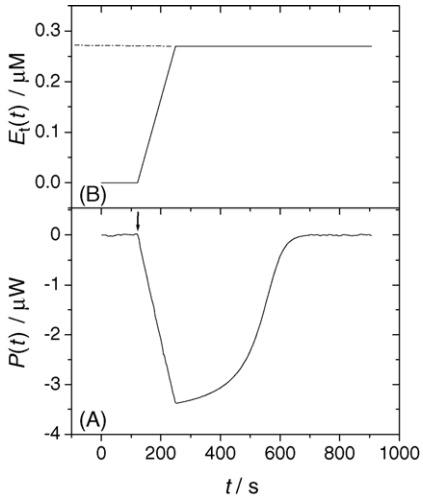

Fig. 1. The isothermal titration calorimetric observation of cellohexaose hydrolysis. (A) The inverting-type endoglucanase catalyzed 1 mM cellohexaose hydrolysis at 30 °C. The substrate solution was 1.0 mM cellohexaose in 20 mM acetate buffer, pH 4.0, loaded in a 1.344 ml ITC cell, and the enzyme solution in the same buffer was loaded in the syringe. After thermal equilibrium was reached at 30 °C, 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) with a time duration of 127 s, giving a final enzyme concentration of 0.27 μM in the ITC cell. (B) The time course of the enzyme concentration of Fig. 1A during the titration. The total enzyme concentration during titration of Fig. 1A is not constant and depends on time. Starting with 122 s the enzyme concentration continues to increase for the next 127 s. So at 249 s, the enzyme concentration reaches its final value of 0.27 μM.

the gradual increase of enzyme concentration. The reaction preceded to the maximal rate at around at 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.

The power resulting from the enzymatic reaction in ITC cell was calculated easily from the data as $\delta P(t) = P(t) - P(\infty)$. By our extended method, we could analyze the complete set of hydrolysis data, including the data on hydrolysis during the titration. Using the data in Fig. 1A, the corrected compensation power of the hydrolytic reaction was monitored as a function of time, and the results are shown in Fig. 2A. The data within the arrows represent the compensation power of hydrolysis heat during titration. The observed total heat was calculated from Fig. 2A using Eq. (16) and monitored as a function of time as shown in Fig. 2B. The Lineweaver–Burk plot of calorimetric observables is shown in Fig. 2C. The linear relationship between the reciprocals of the two variables clearly indicated that this reaction is well approximated by a simple Michaelis–Menten mechanism. The rough estimates of P_{max} and Q_M from the linear line fitted to the data were −3.82 μW and −90.8 μJ, respectively. Using these two values and that for δQ_0 from the data in Fig. 2B, the non-linear least-squares method refined these parameters to fit the experimental data, $P(t)$. Fig. 2D plots the observed power of total experimental data and the best-fitting theoretical function of a simple Michaelis–Menten model. This plot also shows that the model well approximates the endoglucanase-catalyzed hydrolysis reaction of cellohexaose.

In the case of the exothermic reaction, $\delta P(t)$ and $\delta Q(t)$ are negative, as seen in Fig. 2A and B. This is indicated from Eqs. (1) and (2) with negative ΔH, the enthalpy change accompanying the reaction. In Fig. 1A, ΔH for the molar reaction enthalpy equaled the total area under the curve divided by the amount of substrate hydrolyzed in the ITC cell. From the fitted model of Fig. 2D, the most probable values and estimated errors for P_{max}, Q_M, and δQ_0 were determined to be -3.72 ± 0.02 μW, -91.9 ± 1.4 μJ, and -1137.9 ± 4.0 μJ, respectively. Because δQ_0 represents the total hydrolysis heat as mentioned in Section 2, ΔH was calculated to be 847 J mol^{-1} by using the most probable value of δQ_0. From ΔH and the most probable 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. (11) and (12).

3.2. HPLC analysis of the hydrolysis of cello-oligosaccharides

Cellohexaose hydrolysis catalyzed by the inverting-type endoglucanase was also analyzed by HPLC, as shown in Figs. 3 and 4. Considering the ITC data of the hydrolysis observed in Fig. 1A, normal-phase HPLC analysis was conducted to identify and quantify the hydrolysis products. Under the same conditions as used in the ITC experiment shown in Fig. 1A, a series of HPLC experiments were carried out as described previously [13]. A mixture containing 1.0 mM cellohexaose and 0.27 μM the inverting-type endoglucanase was incubated at 30.0 °C. During the incubation, aliquots of the reaction mixture were analyzed at intervals for reaction products by HPLC. Fig. 3 represents the HPLC chromatograms of the hydrolysis products of 1 mM cellohexaose. The 0 min incubation indicates that just after mixing the enzyme and substrate. An aliquot was collected, the enzyme-catalyzed reaction was stopped, and the hydrolysis products were analyzed. The peak areas represent the concentrations of the respective products, and these concentrations were determined by comparison with the standard for cello-oligosaccharides. At 2 and 5 min after the start of the reactions, about 60 and 85% of cellohexaose was hydrolyzed, respectively, and in both the cases almost equal amounts of two products, cellobiose and cellotetraose, were produced. Even 13 min of the inverting-type endoglucanase-catalyzed cellohexaose reaction, appearance of only two products indicated that possibility of further hydrolysis of the products were negligible.

In Fig. 1A, the duration of time used to analyze the hydrolysis data was around 13 min, and the cleavage pattern of cellohexaose in the HPLC analysis shown in Fig. 3 clearly

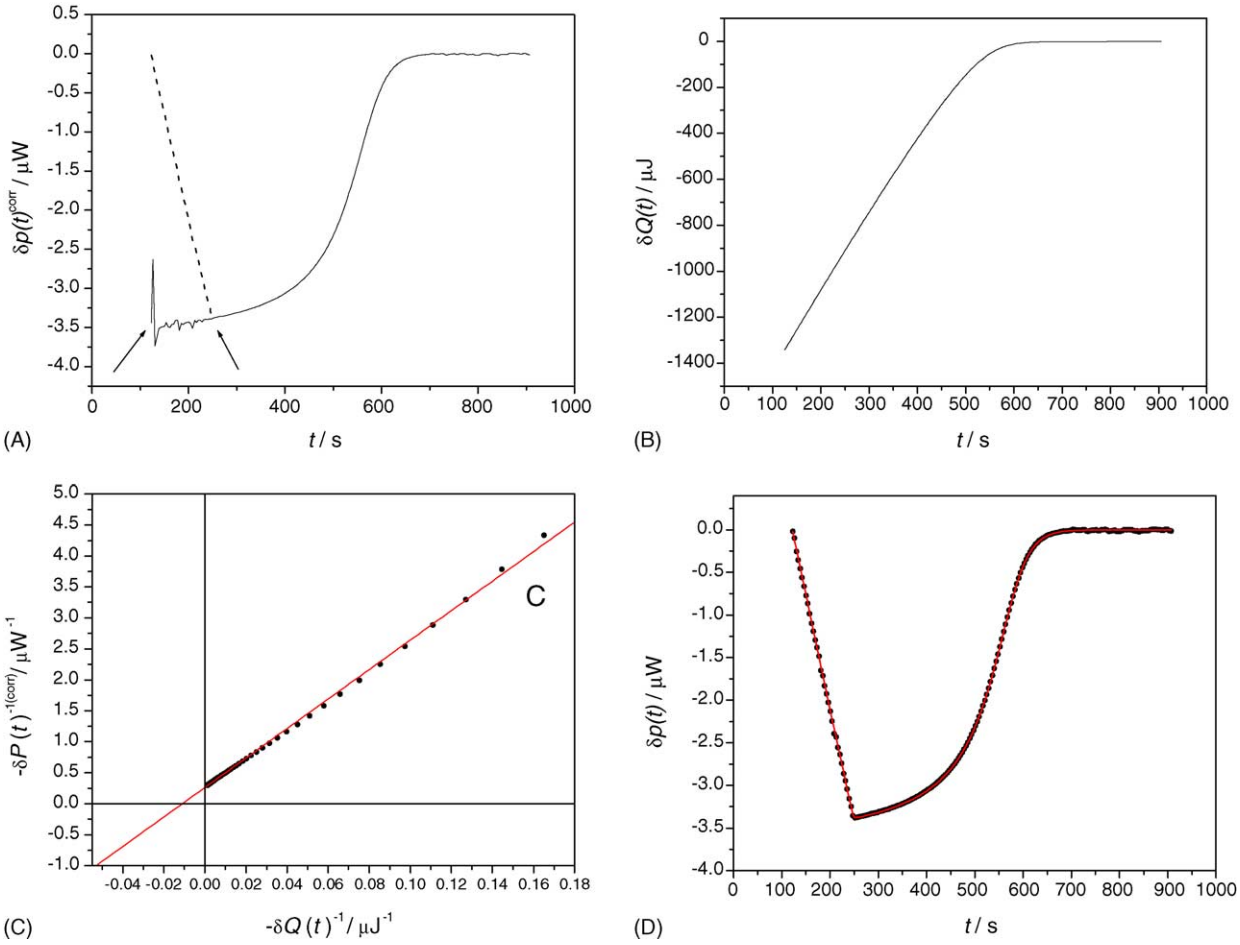

Fig. 2. Experimental ITC data of the inverting-type endoglucanase-catalyzed cellohexaose-hydrolysis of Fig. 1A following the simple Michaelis–Menten model. (A) The corrected compensation power of hydrolysis reaction was monitored as a function of time (represented by a solid line). The dashed lines represent the experimental ITC data during the enzyme injection and the compensation power that had been corrected for the hydrolysis reaction during enzyme injection, representing the deviated part of the solid line between the two arrows. (B) The observed total heat was calculated from Fig. 2A using Eq. (16). (C) The Lineweaver–Burk plot of calorimetric observables, $\delta P(t)^{corr}$ and $\delta Q(t)$ (using the data in (A) and (B)). (D) The plotted points were the observed power of hydrolysis reaction, and the solid line represented the theoretical function on a simple Michaelis–Menten mechanism.

demonstrated that only two products, cellobiose and cellotetraose, appeared during the 13 min of hydrolysis. This result indicated that the observed heat of Fig. 1A was produced from the hydrolysis of one glycosidic bond, and cellohexaose was completely hydrolyzed to cellobiose and cellotetraose in the ITC cell under these experimental conditions.

The time course of cellohexaose hydrolysis in HPLC can be confirmed from the enzymatic parameters obtained from ITC. The integral form of the Michaelis–Menten equation allowed us to estimate the substrate concentration as a function of time during the enzyme-catalyzed reactions. $\delta Q(t)$ was estimated using the ITC data analysis program with the kinetic parameters shown in Fig. 1A and the δQ_0 value for 1 mM cellohexaose. Because $\delta Q(t)$ is proportional to $S(t)$ indicated from Eq. (2), the HPLC analysis of 1 mM cellohexaose depletion as a function of time should be compared with estimated $S(t)$ progress curves as shown in Fig. 4. The comparison clearly showed that the time course of the disappearance of cellohexaose observed in HPLC experiments was in good agreement with the enzyme-catalyzed hydrolysis reaction as observed by ITC.

We also investigated the hydrolytic activity of the inverting-type endoglucanase on other oligosaccharides (G_2–G_5) by HPLC and ITC (data not shown). This endoglucanase was found to degrade cellopentaose considerably more slowly than it degrades cellohexaose. Normal-phase HPLC analysis of the reaction products revealed that cellopentaose was hydrolyzed to cellotriose and cellobiose. As observed previously [13], the comparison of ITC analyzed Michaelis constants of this enzyme for cellohexaose and cellopentaose, at the same substrate concentration, 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. The hydrolysis cleavage pattern of HPLC analysis suggested that more than one glycosidic bond of cellohexaose was

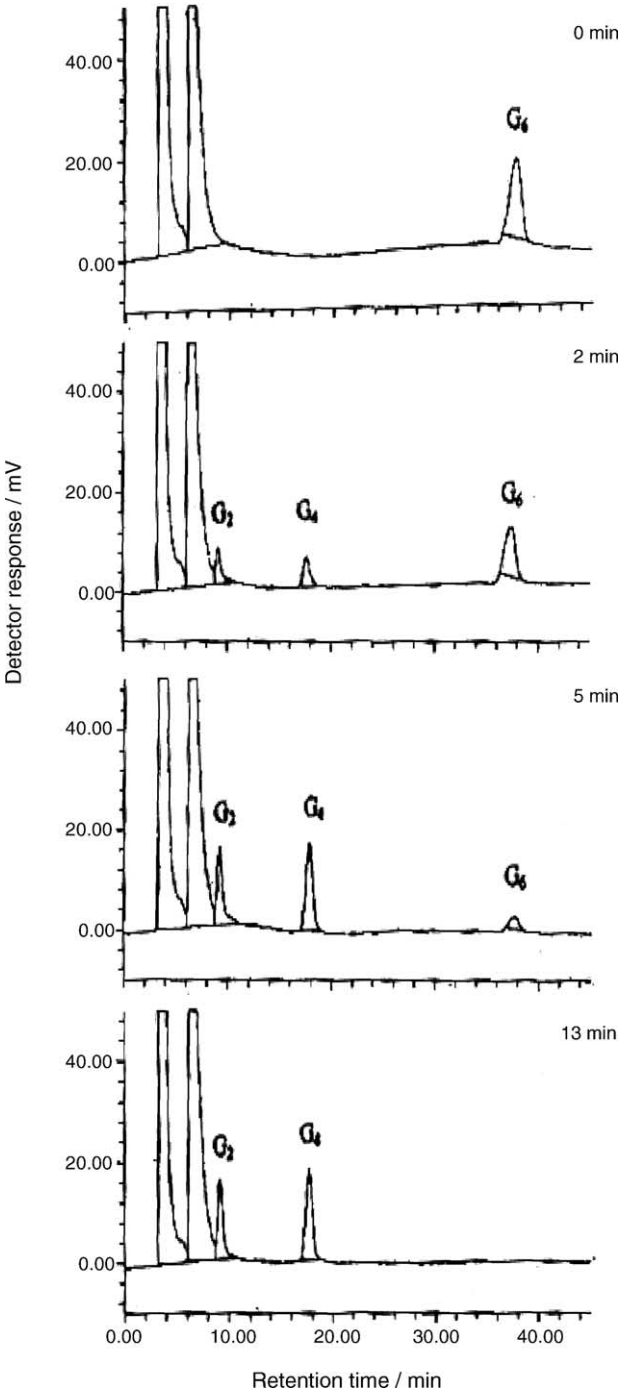

Fig. 3. The HPLC chromatograms showing the hydrolysis products formed by the inverting-type endoglucanase following time course incubation with cellohexaose. The reaction conditions were identical to those shown in Fig. 1A. In the reaction mixture the cellohexaose concentration was 1 mM and the enzyme concentration was 0.27 μM. The enzyme-catalyzed reactions were terminated and hydrolysis products were analyzed as described previously [13]. G_2, G_4 and G_6 represent cellobiose, cellotetraose and cellohexaose, respectively. In each chromatogram, the large two initial peaks represent the detector response for buffer and HCl.

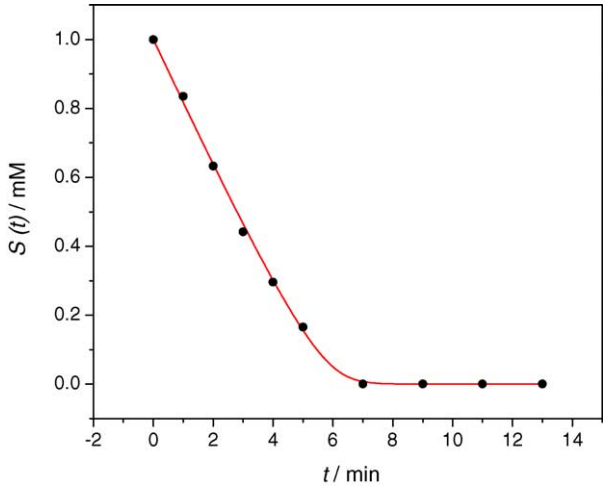

Fig. 4. The time course of the inverting-type endoglucanase-catalyzed cellohexaose hydrolysis in HPLC. It agrees well with the estimated progress curve calculated from the kinetic parameters of Fig. 1A. The closed circles represent the experimental cellohexaose concentration in HPLC and the solid line represents the progress curve derived from Fig. 1A.

hydrolyzed simultaneously from the initial stage, which was considered to make the hydrolysis data analysis very complex and thus eliminate the possibility of using cellohexaose as a suitable substrate for activity observation of the retaining-type endoglucanase by ITC. Like the inverting-type endoglucanase, two major products, cellobiose and cellotriose, were produced when the retaining-type endoglucanase hydrolyzed cellopentaose. Although very negligible amounts of cellotetraose appeared during the enzymatic reaction, it was thought that this minor hydrolysis pathway did not have any major effect on precise measurement of the retaining-type endoglucanase kinetics by ITC using cellopentaose as a substrate. In fact, with an almost identical shape of Fig. 1A, the observed ITC data of the retaining-type endoglucanase-catalyzed cellopentaose hydrolysis (data not shown) at pH 4.0 could be analyzed clearly and simply by our modified analysis based on the simple Michaelis–Menten mechanism.

Virtually no cellobiose (G_2) was hydrolyzed by either of the enzymes, and the hydrolysis rates of cellotriose (G_3) and cellotetraose (G_4) for both the enzymes were too slow to observe by ITC. This was expected to simplify the hydrolysis of cellopentaose and cellohexaose, because further hydrolysis of reaction products could be ignored in the time range used to analyze the hydrolysis data. The HPLC analysis suggested that the retaining-type endoglucanase hydrolyzed cellotetraose (G_4) faster than the inverting-type endoglucanase using in this study.

3.3. Inhibition of endoglucanase activity

Not all cellulases show inhibition behavior, and the nature of substrates has a profound effect on cellulase inhibition. X-ray crystallography showed that a cellulase can bind the substrate and/or products in its substrate-binding sites [27].

This clearly indicates that cello-oligosaccharides become inhibitors as well as substrates. We have previously shown that cellulase activity decreased remarkably when the substrate concentration increased, and we described this inhibition behavior using the term substrate/product inhibition [13]. In the present study, we characterized the apparent inhibition properties by determining whether this substrate concentration dependence on enzyme activities was due to the inhibition exerted by substrate or reaction products. The kinetic parameters of the inverting-type endoglucanase were evaluated by ITC at pH 4.0, using the substrate, cellohexaose, at four different concentrations. Although the k_{cat} values were found to remain constant, the increase in the substrate concentration was accompanied by an increase in the apparent K_M values. Fig. 5 shows that the apparent K_M values increased linearly as the initial cellohexaose concentration increased. Using Eq. (21), the apparent competitive inhibition constant, K_i, at pH 4.0 was found to be 424 ± 32 μM:

$$K_M^{app} = K_M^0 \left(1 + \frac{S_0}{K_i}\right), \quad (21)$$

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

The competitive inhibition of cellulase activity by hydrolysis products has long been studied [28–32], and product inhibition can occur for both soluble and insoluble substrates. In an attempt to evaluate the possible contribution of hydrolysis products to the total inhibition of the inverting-type endoglucanase activity, a comparative analysis of the kinetic parameters of enzyme-catalyzed cellohexaose reactions was made in the absence and presence of products. Table 1 summarizes the kinetic parameters of the inverting-type endoglucanase

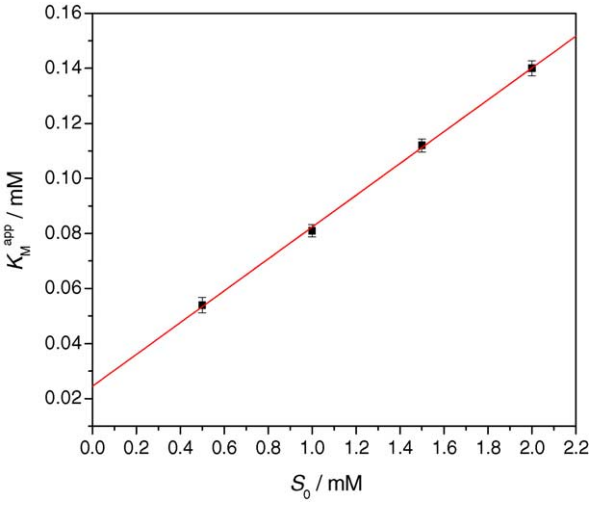

Fig. 5. Substrate concentration dependence of K_M values of inverting-type endoglucanase observed in ITC at pH 4.0. Four different concentrations of cellohexaose (0.5, 1.0, 1.5 and 2.0 mM) were used to measure the apparent K_M values of the enzyme. The apparent K_M values vs. substrate concentrations were plotted. Using Eq. (21), K_M^0 and K_i values were determined to be 25 ± 1 and 424 ± 32 μM, respectively.

Table 1
ITC-evaluated kinetic parameters of the inverting-type endoglucanase in the absence and presence of the hydrolysis products

Substrate	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($mM^{-1}\,s^{-1}$)
a	0.081 ± 0.001	12.1 ± 0.1	149 ± 3
b	0.083 ± 0.002	12.1 ± 0.1	146 ± 5
c	0.087 ± 0.002	12.2 ± 0.1	140 ± 4

a: 1 mM G_6; b: 1 mM G_6 containing 1 mM G_2; c: 1 mM G_6 containing 1 mM G_4. Individual effects of two hydrolysis products, cellobiose and cellotetraose, on the kinetic parameters of enzyme catalyzed cellohexaose reaction are shown. Kinetic parameters of Fig. 1A were used as the data for the substrate only. ITC conditions were same for all the experiments (20 mM acetate buffer, pH 4.0, 30 °C).

as evaluated on cellohexaose with and without the hydrolysis products. Although there was no major change of k_{cat} values among the three observations, a slight and gradual increase of K_M values was observed when cellohexaose was hydrolyzed separately in the presence of cellobiose and cellotetraose rather than using the cellohexaose in the absence of products. As compared with the value of the observed inhibition constant, 424 μM, the slight increase of observed K_M values for cellohexaose in the presence of the products diminished the possibility of any significant product inhibition for this enzyme.

Because Fig. 5 clearly shows that the increased substrate concentration had a significant effect on the activity, and the products made only a very small contribution to the inhibition, the observed inhibition was considered to have occurred due to the substrate. We should therefore use the term "substrate inhibition" to refer to the apparent decrease of reaction rate which accompanies the increase of substrate concentration. This term has frequently been used for the hydrolysis of insoluble substrates catalyzed by cellulases [33–35], and different substrates show different substrate inhibition properties [35]. The present study clearly indicates the substrate inhibition for the cellulase-catalyzed hydrolysis of oligosaccharides.

3.4. ITC observation of endoglucanase-catalyzed cello-oligosaccharides hydrolysis at pH 7.0

ITC observation of the cello-oligosaccharides hydrolysis at pH 7.0 was somewhat different from that observed at pH 4.0. The activities of both the inverting-type and retaining-type endoglucanases were evaluated by ITC as observed in Figs. 6 and 7, respectively. As compared with Fig. 1A, the main difference in the ITC curves of these two figures was that, after hydrolysis, the baseline was shifted from the pre-injection level. In order to determine the possible causes of this discrepancy between the ITC-observed hydrolyses at the two different pHs we first considered that the hydrolysis products of substrates (cellohexaose for inverting-type and cellopentaose for 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

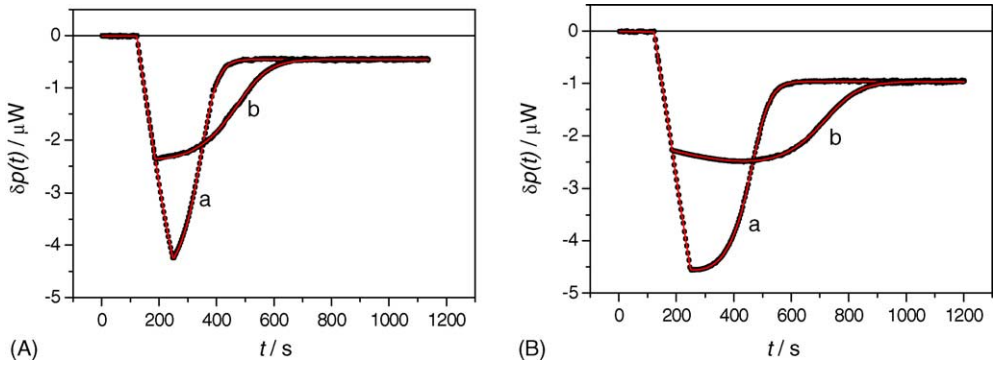

Fig. 6. ITC observation of cellohexaose hydrolysis catalyzed by the inverting-type endoglucanase at 30 °C, pH 7.0. (A) In two separate experiments, 0.5 mM cellohexaose is hydrolyzed by two different concentrations of enzymes. After thermal equilibrium was reached, 60 and 30 μl of enzyme solutions were injected at 122 s into the ITC cell with the duration of 127 and 63.5 s, respectively. Therefore the two ITC curves (indicated by a and b) differed only in their final enzyme concentrations. The enzyme concentration in the syringe for both cases was 12.6 μM, and the final enzyme concentration for curve a and b were calculated to be 0.538 and 0.275 μM, respectively. In both curves the dotted points were the observed power and the theoretical model represented by solid lines. (B) With identical conditions of (A) 1 mM cellohexaose was hydrolyzed by two different concentrations of enzyme in separate ITC experiments. The final enzyme concentrations of the curves a and b of this figure were the same of those used in curves a and b of (A).

that, in the time range used to analyze ITC data at pH 7.0, the hydrolysis patterns were the same for both enzymes as those at pH 4.0. Furthermore, if this shift was due to the hydrolysis reaction, it would be expected to be dependent on the concentration of the enzyme. The finding that the shift was clearly dependent on the substrate concentration and clearly not dependent on the enzyme concentration, as shown in Fig. 6, indicates that the heat is not related to the enzyme-catalyzed reaction but the product-related reaction. The probable cause of the shift at pH 7.0 was the mutarotation reaction of hydrolysis products.

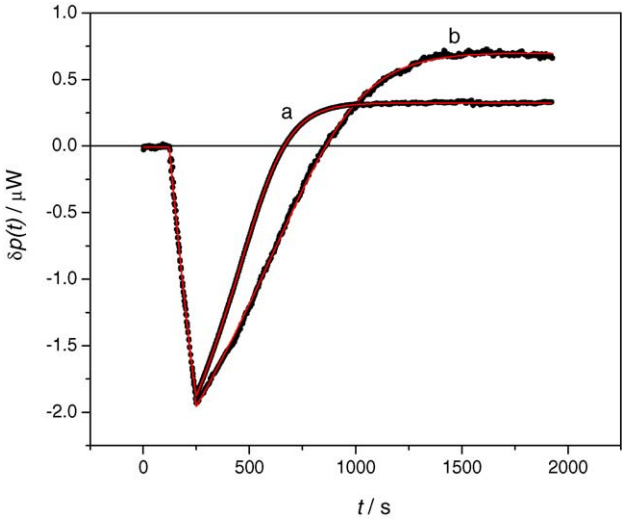

Fig. 7. ITC observation of cellopentaose hydrolysis by the retaining-type endoglucanase at 30 °C at pH 7.0. Two concentrations of cellopentaose (0.5 and 1.0 mM) were hydrolyzed by a fixed amount of the enzyme in two different ITC experiments. After thermal equilibrium, for both the cases, 60 μl of enzyme solution whose concentration was 5.0 μM in the syringe was injected at 122 s giving a final enzyme concentration of 0.214 μM in the ITC cell. Two curves were designated, a and b, for the substrate concentration 0.5 and 1.0 mM, respectively. Dotted points were observed powers from experimental data and solid lines represented the best-fitted theoretical model.

In general, mutarotation of sugars can be defined as the interconversion of anomeric forms accompanied with the change in optical rotation. Some previous studies have tried to describe the mutarotation of hydrolysis products of cello-oligosaccharides catalyzed by cellulases [30,36–38]. On the other hand, attempts have been made to observe the mutarotation heat of various types of sugars by calorimetry [20–22,39–41]. However, to the best of our knowledge, our present study is the first to apply ITC for continuous observation of both the cellulase-catalyzed hydrolysis of cello-oligosaccharides and the mutarotation of products. In order to observe the expected contribution of the mutarotation heat of hydrolysis products and simultaneously evaluate the kinetic parameters from Figs. 6 and 7, an extended program was used as described in Section 2.

The inverting-type and retaining-type endoglucanases have different catalytic mechanisms, i.e., inversion and retention of the configuration of the β-glycosidic bond of the substrate, 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 of the α- and β-anomeric forms. In Fig. 6, the baseline deviated downward after hydrolysis, indicating the exothermic heat produced after hydrolysis of the substrate. It seemed probable that this was exothermic heat generated from the mutarotation. In order to evaluate the kinetic parameters from the hydrolysis reactions in Fig. 6, we must separate the hydrolysis heat from the heat contributed by mutarotation of products. The rough estimates of the calorimetric observables were found to be suitable to fit the experimental data. From the least-squares analysis of the data, we obtained the refined val-

Table 2
Comparative analysis of the kinetic parameters of the inverting-type and the retaining-type endoglucanases and expected mutarotation contributions of hydrolysis products at pH 7.0, as determined from the findings in Figs. 6 and 7

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

Because the concentrations of enzyme 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).
[a] Mutarotation contributions of products.

ues of δQ_0 (the total hydrolysis heat), P_{max}, Q_M and the contribution of mutarotation heat of the hydrolysis products. The molar enthalpy change, ΔH, accompanying the hydrolysis reactions was calculated from δQ_0, and the kinetic parameters of the inverting-type endoglucanase-catalyzed cellohexaose reactions at pH 7.0 were evaluated using the values of P_{max} and Q_M. The difference observed between Fig. 6A and B was the amount of deviation of the baseline after hydrolysis, and this difference can be approximated to be proportional to the initial substrate concentrations, as mentioned in Section 2.

Based on the observation of mutarotation heat in ITC, contrasting result obtained when cellopentaose was hydrolyzed by the retaining-type endoglucanase at pH 7.0. The hydrolysis products are retained as a β-configuration, and then β to α mutarotation occurs to attain equilibrium. In Fig. 7, two different experimental ITC curves were plotted (a and b), and these experiments were designed and carried out under the same ITC conditions and the same enzyme concentrations. The only difference between them was the substrate concentrations (0.5 mM for curve a and 1.0 mM for curve b) in the ITC cell. In both the cases of Fig. 7, the baseline deviated upwards indicating that heat was absorbed after hydrolysis, and this endothermic heat was predicted to be due to the contribution of mutarotation of products. As expected, in a comparative analysis of ITC observations with different concentrations of enzyme (data not shown), this mutarotation heat was not found to be dependent on the final enzyme concentrations in the ITC cell.

Table 2 summarizes the kinetic parameters of the two types of endoglucanases as well as the estimated mutarotation contributions of hydrolysis products as evaluated by ITC from Figs. 6 and 7. In addition to the kinetic parameters, two very valuable findings on the mutarotation heat were shown. The first one was the fact that the inverting-type and retaining-type endoglucanases produced different signs of the mutarotation heat with each other. Namely the exothermic and endothermic heat produced from the mutarotation of hydrolysis products catalyzed by inverting-type and retaining-type endoglucanase, respectively. For both types of enzymes, it was quantitatively shown that the mutarotation heat was dependent on the initial substrate concentrations, and hence the magnitude of the contribution of the mutarotation is almost doubled when the substrate concentration is doubled for both types of enzymes.

4. Discussion

It has been well established that calorimetry has numerous advantages when compared with the traditional spectroscopic method [4–6,12]. 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 E.C classifications, and the simple and complex kinetics and calorimetrically evaluated kinetic parameters agree well with the published values in most cases [6]. In two previous studies, we have successfully applied this method to evaluate the protease [12] and cellulase kinetics [13] using the usual data analysis method. The present method of ITC data analysis was developed in view of a new concept: the time dependence of enzyme concentration during the titration, as shown in Fig. 1. The new data analysis method was characterized by the point that it could evaluate hydrolysis heat during titration (Fig. 2A), and thus that it could evaluate the kinetics of enzyme-catalyzed reactions more precisely by using a more complete set of data points, including the hydrolysis data during enzyme titration. Furthermore, the efficacy and acceptability of this method have been confirmed by means of the experimental results obtained in the current study.

Figs. 1 and 2 demonstrate the application of the new data analysis method of ITC to monitor endoglucanase-catalyzed cellohexaose hydrolysis, and the kinetic parameters, K_M and k_{cat}, obtained from the calorimetric observables, clearly indicating that the hydrolysis reaction was well approximated by a simple Michaelis–Menten equation. In addition, the comparison between ITC observation and the HPLC analyzed cellohexaose hydrolysis (Figs. 3 and 4) strongly suggested that the hydrolysis heat of a single glycosidic bond was observed in ITC, and the time course of the hydrolysis reaction followed the Michaelis–Menten kinetics.

Because no difference of initial and final baselines in Fig. 1A, only the hydrolysis heat without the mutarotation was observed at pH 4.0. From this fact, 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 in the time range used to analyze the enzymatic reaction in this study. This result also agrees reasonably well with the previous observations [22,42] that the mutarotation rate greatly depends on pH, and in acidic pH this rate becomes very slow.

The hydrolysis reaction rate was slower at pH 7.0 than at pH 4.0, as determined from the kinetic parameters measured at the two pHs. The k_{cat} and K_M were affected by pH very much. The value of k_{cat} was found to be decreased and that of K_M to be increased at pH 7.0.

At the same time, Figs. 6 and 7 clearly indicate that mutarotation of the hydrolyzed products had a significant effect on the observed endoglucanase-catalyzed hydrolysis of cello-oligosaccharides at pH 7.0. Therefore, it is likely that the mutarotation rates become considerably high at neutral pH as compared with pH 4.0. In this study, one of the noteworthy findings was the successful separation of hydrolysis heat from the heat contributed by mutarotation of the products at neutral pH, and the successful evaluation of kinetic parameters of enzyme-catalyzed reactions even at the neutral pH.

Moreover, Table 2 clearly shows that the mutarotation heat of the products depends on the initial substrate concentrations. In addition, the opposite signs in the mutarotation for the two types of enzymes can be explained in view of the difference in the anomeric forms of the hydrolysis products and by the direction of subsequent mutarotation. An inverting-type endoglucanase produced products of α-anomeric forms, and α to β mutarotation occurred in this case. On the other hand, hydrolysis products for retaining-type endoglucanase mutarotate from β to α, the opposite direction of the former enzyme. In general, the ratio of anomeric forms of sugars in equilibrium mixtures depends on the sugar types and also affected by the temperature of the solution. The equilibrium composition of β-form of glucose and cellobiose was reported 63.6 and 64.4%, respectively, at 24.5 °C [21]. The calculated values of equilibrium composition of two anomeric forms of various sugars were compared with values those by NMR experiment in [43], where the fraction of α-D-glucose was reported to be 36% for both the cases. Since, in our case, among the hydrolysis products, cellobiose was the potential candidate, predicted to contribute mutarotation heat, the assumption that the ratio of the α- and β-anomeric forms of the hydrolysis products was 4/6 in the equilibrium mixture may not seem unreasonable. The most probable reason for the exothermic mutarotation produced by inverting-type endoglucanases was all products with α-forms underwent mutarotation to β-forms to attain equilibrium, and as a result heat was released. The exactly opposite phenomenon was considered to occur in the case of retaining-type endoglucanase, where the mutarotation heat was found to be absorbed.

The mutarotation of α- and β-D-glucose have been investigated more extensively than those of other sugars. The heats of mutarotation of α- and β-D-glucose were precisely measured previously and found to be negative (exothermic) and positive (endothermic), respectively [41]. Since our results are in satisfactory agreement with this published report qualitatively, it can be predicted as a remarkable finding that the inverting-type endoglucanase hydrolysis is followed by exothermic mutarotation heat of the products while for retaining-type endoglucanase, endothermic heat observed from the hydrolysis products.

From Eq. (19), we can estimate the rate constant of the mutarotation from the observed mutarotation power, P_{mut}^{max}, if the molar enthalpy of the mutarotation is known. Although the enthalpy may depend on the hydrolyzed product and the exact value is not clear at this stage, we may estimate the rate constant using the enthalpy of cellobiose [21], $-1.8\,kJ\,mol^{-1}$. With our substrate concentration and the cell volume, the rate constants for the α-anomer and the β-anomer products in Table 2 were calculated to be $3.8 \times 10^{-4}\,s^{-1}$ and $2.8 \times 10^{-4}\,s^{-1}$, respectively, at pH 7.0 and 30 °C. These values are considered to be reasonable, since the apparent rate constant of the mutarotation of cellobiose were previously reported to be $8.8 \times 10^{-5}\,s^{-1}$ at pH 5.5 and 25 °C [21], and the mutarotation rate increases by increasing pH and temperature. The ratio of the observed rate constants of both anomers is also consistent with the ratio of the fractions of the anomers in equilibrium.

In summary, the experimental results obtained and described in this study demonstrate that the capability of the new data analysis method for precise evaluation of cellulase kinetics has increased the potential use of ITC as a general analysis tool for many kinds of enzymes. Furthermore, the mutarotation heat has a great importance to determine the hydrolysis mechanism of the endoglucanases.

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