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Precise and continuous observation of cellulase-catalyzed hydrolysis of cello-oligosaccharides using isothermal titration calorimetry

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Abstract

Heat is a characteristic of all chemical reactions, and isothermal titration calorimetry (ITC) provides a possible way to continuously detect the heat from catalytic reactions with high sensitivity and reproducibility. Cellulase, the enzyme of glycosyl hydrolase, catalyzes the cleavage of β-1,4 glycosidic bonds in cellulose. In this paper, ITC was applied to evaluate cellulase activity using cello-oligosaccharides as substrates. The hydrolysis heat of a single glycosidic bond of the substrate was successfully detected by combining ITC and normal-phase HPLC, and the time course of the enzymatic reaction was monitored continuously by ITC. The enzymatic parameters k_{cat} and K_M, obtained from calorimetric observables, clearly indicated that the reaction was well approximated by a simple Michaelis–Menten equation under the experimental conditions of this study. The normal-phase HPLC analysis was combined with the ITC approach to observe hydrolysis patterns and was found to be an effective and precise way to evaluate the activity of cellulase against cello-oligosaccharides.
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1. Introduction

Quantitative evaluation of the catalytic activity of enzymes is inevitably important in order to reveal the enzymes' reaction mechanisms and to use the catalysts most effectively. With the accumulation of genetic information and the simplification of methods for creating site-directed mutations, an increase in comparative enzyme analysis is anticipated. A generally applicable analysis method that is precise, rapid, and simple will help in the effort to characterize enzymes completely.

Since all enzymatic reactions proceed with an exchange of heat generally, some studies have employed calorimetry to monitor these reactions [1–6]. ITC provides a way to detect the catalytic reaction heat and thereby the kinetics of an enzyme with high sensitivity and reproducibility. Two calorimetric observables, the compensation power and its integrals, can be determined directly and precisely by titration calorimetry [6]. In the hydrolytic condition, the combination of a calorimetric Lineweaver–Burk plot and non-linear least-squares method with these two observables was found to be effective to determine the enzymatic parameters precisely [6]. Because it allows the direct observation of reaction rates, calorimetry is expected to provide an effective and precise way to evaluate enzyme activity. The present study applies ITC to evaluate the activity of an enzyme, cellulase, which hydrolyzes cellulose, a polymer of β-1,4-linked glucose residues. Cellulose is the major polysaccharide component of plant cell walls and the most abundant biopolymer.

Cellulose is degraded enzymatically by a wide variety of plant, bacterial, and fungal species that possess endoglucanases, cellobiohydrolases, and β glucosidases that synergistically facilitate the complete cleavage of the cellulose β-1,4 glycosidic bonds [7–9]. However, the choice of the most appropriate substrate for the kinetic analysis of cellulase has always been a huge problem. Steady-state kinetics of the hydrolysis of a well-defined soluble substrate is a precious tool for understanding the mechanism underlying cellulolytic enzymes. Cello-oligosaccharides that have a chromogenic group such an aglycon or radioactive-labeled

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cello-oligosaccharides, are well known substrates for steady-state kinetic studies of cellulases [10–12]. The catalytic activity of a wide range of cellulases has been investigated using reduced cello-oligosaccharides as substrates with the coupled assay of cellobiose dehydrogenase [13].

The present work applies ITC to an approach for evaluating the kinetics of cellulase-catalyzed hydrolysis of cello-oligosaccharides. We also conduct a combined analysis of ITC and normal-phage HPLC to understand the cleavage patterns of cello-oligosaccharides and thus to evaluate more precisely the time course of enzymatic reactions observed by ITC. The substrate and/or product inhibition was also checked by observing the dependence of the apparent Michaelis constant on the substrate concentration in this paper.

2. Materials and methods

The enzyme used in this study was a fungal cellulase kindly supplied by Meiji Seika Kaisha Ltd., Japan. All enzyme experiments were performed in 20 mM acetate buffer, pH 4.0, at 30.0 °C. Enzyme powder was solved in buffer solution and filtered through a membrane filter (Millipore, USA) whose filtered molecular weight was 100 kDa. The enzyme solution was concentrated and dialyzed by exchanging the buffer solution using the Amicon ultrafiltration system (Millipore) with an ultrafiltration membrane polyethersulfone with a 10 kDa cut-off. The enzyme concentration was determined by monitoring the absorbance with a UB-35 spectrophotometer (Jasco Co., Japan) using an extinction coefficient of 93.6 $cm^{-1}\,mM^{-1}$ at 280 nm. The coefficient was determined by evaluating the enzyme concentration through the bicinchoninic acid (BCA) method (Pierce Chemical, USA), with bovine serum albumin (BSA) as the standard of protein concentration.

Five cello-oligosaccharides—cellobiose, cellotriose, cellotetraose, cellopentaose, and cellohexaose—were purchased from Seikagaku Co. (Japan) and used without further purification. To identify and quantify the reaction products of each cello-oligosaccharide, a normal-phase HPLC system was used in conjunction with 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). The retention times of the hydrolysis products were compared to standards of cello-oligosaccharides.

An isothermal titration calorimetry (ITC) unit of the MCS system (Microcal, USA) was used with an control software provided by the manufacturer, Microcal Observer 3.0, on an personal computer, Optiplex GX 100 PC (Dell, USA), running Windows 95. The temperature of the titration cell was kept at 30.0 °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 this study, the implementation of ITC to evaluate cellulase kinetics was based on the use of derivative equations that had been successfully applied to evaluate protease activity [6]. Although the calorimetric observables to evaluate the enzymatic parameters were previously discussed in that study, they are presented in simple form below.

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 observables 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).

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

Using Eqs. (1) and (2), the Michaelis–Menten equation and its integral form are formulated as the following equations, respectively:

$$\delta P(t) = \frac{P_{max}\delta Q(t)}{Q_M + \delta Q(t)} \tag{4}$$

$$\frac{1}{P_{max}}[\delta Q_0 - \delta Q(t)] + \frac{Q_M}{P_{max}E_0}\ln\frac{\delta Q_0}{\delta Q(t)} = t - t_0 \tag{5}$$

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

$$P_{max} = V_c \Delta H k_{cat} E_0 \tag{6}$$

$$Q_M = V_c \Delta H K_M \tag{7}$$

where E_0 is the initial enzyme concentration in the mixture, and k_{cat} and K_M are the turnover number and the Michaelis constant, respectively.

Total absorbed heat measured in calorimeter at $t = t_0$.

$$\delta Q_0 = \delta Q(t_0) = V_c \Delta H S_0 \tag{8}$$

where S_0 is the substrate concentration at time t_0. Although t_0 could be set to any time, in this paper it denotes the time at which all of the enzyme solution has been injected into the calorimeter cell.

Eq. (9) can be derived from Eq. (4), which corresponds to the Lineweaver–Burk equation in a traditional way to

determine the enzyme parameters with the reaction rate and substrate concentration.

$$\frac{1}{\delta P(t)} = \frac{Q_M}{P_{max}} \frac{1}{\delta Q(t)} + \frac{1}{P_{max}} \quad (9)$$

From this equation, 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 fitting parameters can be refined by a non-linear least-squares method. With this method the theoretical function can be calculated from Eq. (4), and three fitting parameters, P_{max}, Q_M, and δQ_0, will be adjusted to fit the experimental data directly. The initial values for P_{max}, Q_M are taken from the plot of Eq. (9). That for δQ_0 can be set from the observable $\delta Q_0(t)$ at $t = t_0$.

A FORTRAN program was custom-written using a non-linear least-squares program package, SALS [14]. The modified Marquardt method was used for the parameter refinement.

The HPLC experiments were designed such that the enzyme and substrate were in the same concentrations as they were in the ITC cell and the enzymatic reaction conditions were identical with those used in the ITC experiments. The cello-oligosaccharides were incubated with cellulase, and the hydrolysis reactions were terminated at various intervals by adding HCl to a final concentration of 2.4 M. In order to improve the resolution in the HPLC chromatograms, the acetonitrile solution was mixed with the sample solution at a 1:1 ratio.

3. Results and discussion

Fig. 1 shows a typical time course of the compensation power for a sample cell of the isothermal titration calorimeter. After thermal equilibrium was reached at 30.0 °C, the enzyme solution injection began at 120 s and lasted 127 s. Exothermic heat accompanying the hydrolysis of 1 mM cellohexaose by cellulase was monitored immediately after the enzyme injection was complete. This paper denotes the time 247 s as t_0 (indicated by the arrow in the figure), the hydrolysis starting time. The observed power reached a maximum just at this time, t_0, and the reaction proceeded near the maximal rate (V_{max}) in this stage, then decayed to the baseline as the substrate was depleted completely. When all the

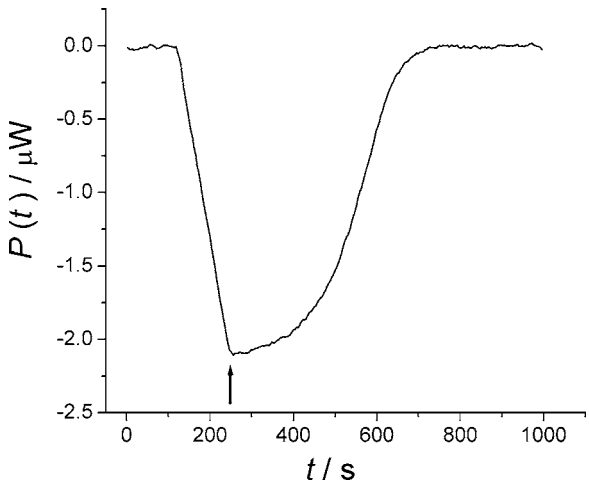

Fig. 1. Isothermal titration calorimetric observation of hydrolysis of a glycosidic bond with cellulase at 30.0 °C. The compensation power was monitored as a function of time with stirring at 41.9 rad s^{-1}. The substrate solution in the cell was 1.0 mM cellohexaose in 20 mM acetate buffer at pH 4.0. The baseline stability was checked before injection. After 120 s, 60 μl of the enzyme solution was added during the next 127 s. Beginning at that point, 247 s, the hydrolysis reaction was analyzed (indicated by arrow). The enzyme concentration of injected solution was 2.57 μM and that of the calorimeter cell was calculated to be 0.11 μM.

cellohexaose was hydrolyzed, the observed power returned almost to the pre-injection level. Considering the hydrolysis ITC data observed in Fig. 1, normal-phase HPLC analysis was conducted to identify the hydrolysis products. Under the same conditions in the ITC experiment shown in Fig. 1, HPLC experiments were carried out as described in Materials and Methods. The HPLC results in Table 1 clearly show that during 10 min of cellulase-catalyzed hydrolysis, cellohexaose was completely hydrolyzed to cellotetraose and cellobiose, and any further hydrolysis could be ignored in this condition. So, the heat of the enzymatic reaction in Fig. 1 corresponds to the hydrolysis of only one glycosidic bond, and the cellulase-catalyzed hydrolysis reaction occurring in the ITC cell can be represented by the following scheme.

$$\text{Cellohexaose} + H_2O \rightarrow \text{cellotetraose} + \text{cellobiose} \quad (10)$$

The power resulting from this enzymatic reaction was calculated easily from the data as $\delta P(t) = P(t) - P(\infty)$. Using the data in Fig. 1, the compensation power of hydrolytic reaction and its integral were monitored as a function of

Table 1
HPLC analysis of hydrolysis products of 1 mM cellohexaose by cellulase

Incubation time (min)	Hydrolysis product (mM)				
	Cellobiose	Cellotriose	Cellotetraose	Cellopentaose	Cellohexaose
0.0	nd	nd	nd	nd	0.98
5.0	0.49	nd	0.52	nd	0.43
10.0	1.02	nd	0.96	nd	nd

The concentrations of reactants and reaction conditions in the HPLC experiments are identical to those shown in Fig. 1. The time, t_0, in Fig. 1 represents 0 incubation time in HPLC analysis (nd: not detected).

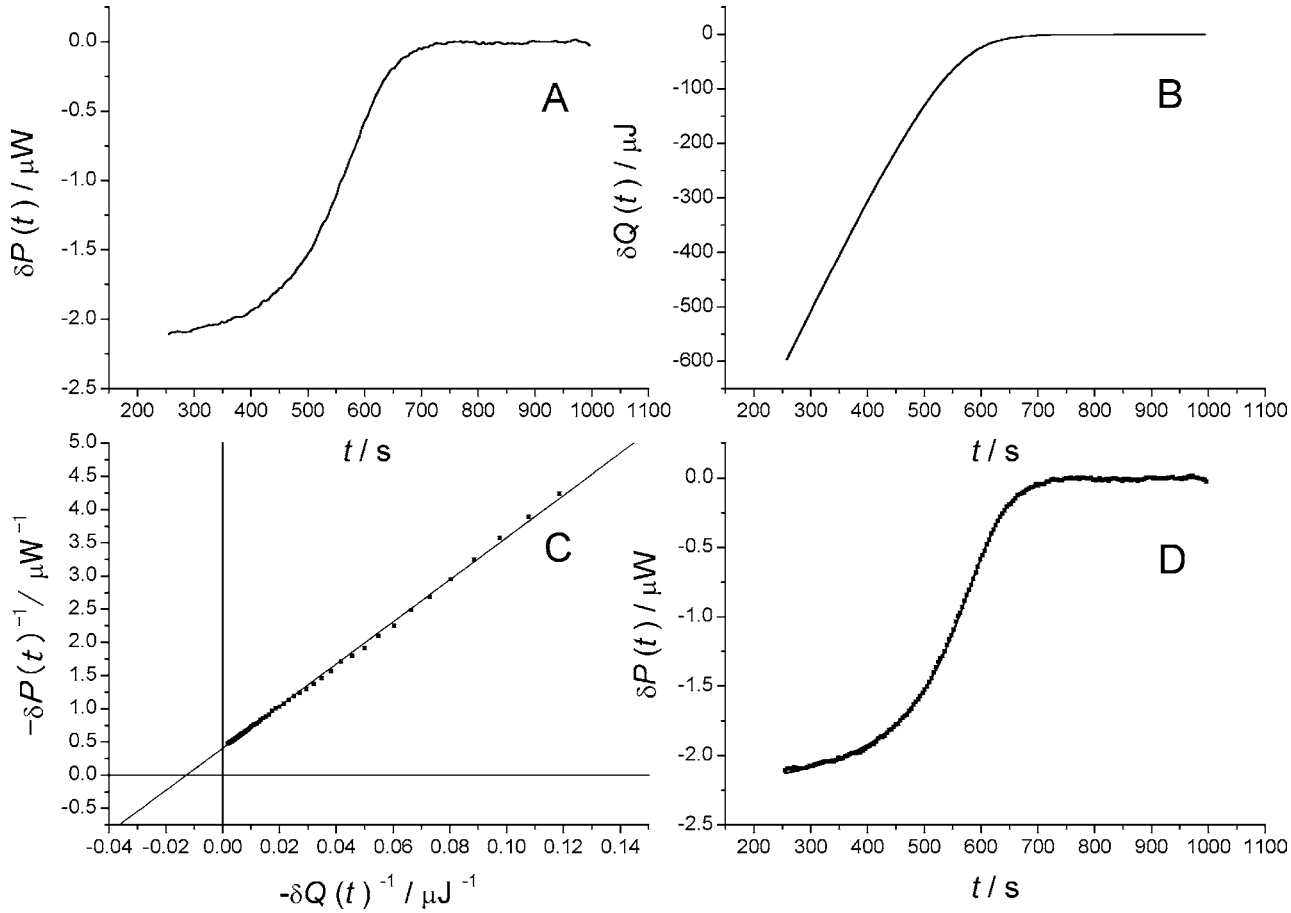

Fig. 2. Experimental ITC data of the cellulase-catalyzed cellohexaose-hydrolysis of Fig. 1 follows the simple Michaelis–Menten model. (A) Compensation power of hydrolysis reaction was monitored as a function of time. (B) The observed total heat was calculated from Fig. 2A using Eq. (3). (C) The Lineweaver–Burk plot of calorimetric observables, $\delta P(t)$ and $\delta Q(t)$ (using the data in Fig. 2A and B). (D) The plotted points were the observed power, and the solid line represents the theoretical function on a simple Michaelis–Menten mechanism.

time and are shown in Fig. 2A and B, respectively. The Lineweaver–Burk plot of calorimetric observables was shown in Fig. 2C. The linear relationship between the reciprocals of the two observables 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 $-2.45\,\mu W$ and $-77.7\,\mu J$, 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 and the best-fitting theoretical function of a simple Michaelis–Menten model. This plot also shows that the model well approximates the cellulase-catalyzed hydrolysis reaction of cellohexaose.

In the case of 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. 1, the ΔH for the molar reaction enthalpy equaled the total area under the curve, namely the total heat produced (Q_T) divided by the amount of substrate hydrolyzed in the ITC cell. The value of Q_T during the hydrolysis of 1 mM cellohexaose was found to be $-755\,\mu J$, and ΔH was calculated to be $-562\,J mol^{-1}$. The most probable values and estimated errors for P_{max}, Q_M, and δQ_0 were determined to be $-2.395 \pm 0.003\,\mu W$, $-76.1 \pm 0.4\,\mu J$, and $-604.3 \pm 0.4\,\mu J$, respectively. From ΔH and the most probable values of P_{max} and Q_M, the k_{cat} and K_M values of cellulase to cellohexaose can be evaluated to be $29.7\,s^{-1}$ and 0.101 mM, respectively, using Eqs. (6) and (7).

Although to our knowledge this is the first report to observe the enthalpy of hydrolysis of one glycoside bond of a cello-oligosaccharide, those of disaccharides were reported in the range of -5 to $+6\,kJ\,mol^{-1}$, including the mutarotation heat, depending on the saccharide type [15]. As our observed enthalpy, $-0.56\,kJ\,mol^{-1}$, is almost the central value in this range, it does not seem to be particularly small. Considering that the absolute value of the present result was almost one-tenth the typical molar enthalpy of hydrolysis, our results, even with the small molar enthalpy, have clearly shown the possibility of using this method to observe the hydrolysis of other saccharides.

We also used ITC to investigate the hydrolytic activity of the cellulase on cellopentaose. Normal-phase HPLC analysis of the reaction products revealed that the cellulase

Table 2
Kinetic parameters of cellulase for cellohexaose and cellopentaose as evaluated by ITC

Substrate	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} mM^{-1})
Cellohexaose	0.101	29.7	294
Cellopentaose	0.765	19.5	25.5

For cellohexaose, the kinetic parameters were obtained from Fig. 1; cellopentaose kinetic parameters were calculated from the ITC experiment in which 1 mM cellopentaose was reacted with 0.56 μM cellulase in the ITC cell. ITC conditions were identical in both experiments (pH 4.0, 20 mM acetate buffer, 30.0 °C).

hydrolyzed cellopentaose to cellotriose and cellobiose. Table 2 summarizes the kinetic parameters of cellulase on cellohexaose and cellopentaose as evaluated by ITC. A comparison of the Michaelis constants of these substrates at the same substrate concentration, 1 mM, suggested that cellopentaose had less affinity to cellulase than cellohexaose had.

Moreover, cellulase activities were found to decrease remarkably when the substrate concentration increased, as shown in Fig. 3. Since the k_{cat} values were found to remain almost constant, we prefer to use the term "substrate and/or product inhibition" to refer to any apparent increase of K_M that accompanies an increase in the substrate concentration. The use of this term allows us to assume that the substrate and/or the reaction products inhibited the enzyme activity in a competitive way. Fig. 3 shows that the apparent K_M values of cellulase increased linearly as the initial cellohexaose concentration increased. Using Eq. (11) the inhibition constant, K_i, at pH 4.0 is found to be 2.4 ± 0.6 mM.

$$K_M^{app} = K_M^0 \left(1 + \frac{S_0}{K_i}\right) \quad (11)$$

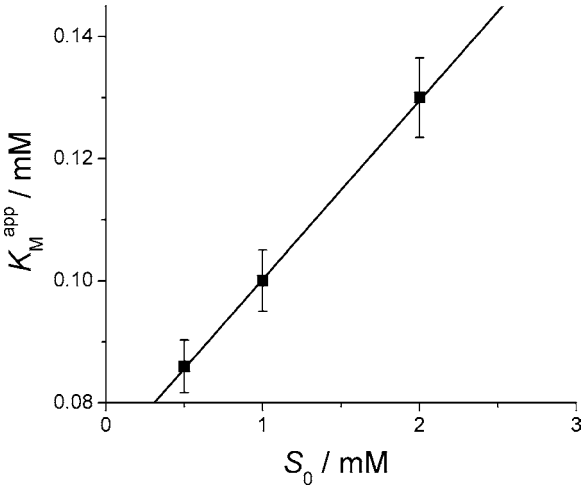

Fig. 3. Substrate concentration-dependence of K_M values of cellulase observed in ITC. Three concentrations of cellohexaose (0.5 mM, 1.0 Mm, and 2.0 mM) were used to measure the apparent K_M values of cellulase. The apparent K_M values were plotted versus substrate concentrations. Using Eq. (11), K_M^0 and K_i values were evaluated to be 71.1 ± 5.8 μM and 2.4 ± 0.6 mM, respectively.

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.

If the product binds more strongly than the substrate—that is, if the inhibition constant, K_i, of the product is smaller than the Michaelis constant for the substrate—it is well known that the time course of the hydrolysis will not be explained by a normal Michaelis–Menten equation. In the present case, Fig. 2C and D strongly suggest that the time course of the hydrolysis occurred as predicted by a normal Michaelis–Menten mechanism, and hence the inhibition constant of the product was larger than the Michaelis constant. Indeed, the observed inhibition constant, 2.4 mM, was much larger than the estimated Michaelis constant, 0.07 mM.

The advantages of calorimetry over the traditional spectroscopic method have long been recognized [3–6]. The calorimetrically determined kinetic parameters of various well-known enzymes agree well with the published values in most cases [5]. To the best of our knowledge, however, the present work is the first to apply ITC to measure cellulase kinetics continuously. Our study clearly shows that the reaction rate of cellulase catalysis can be precisely and easily obtained by ITC, and that the kinetic parameters, K_M and k_{cat}, obtained from calorimetric observables, clearly indicate that the enzyme-catalyzed hydrolysis of cello-oligosaccharides is well approximated by a simple Michaelis–Menten equation. Cellohexaose was considered to be a good substrate for the calorimetric determination of the cellulase activity.

This cellulase hydrolyzed cellotetraose and cellotriose too slowly to allow observation by ITC (data not shown). This is expected to simplify the hydrolysis of cellohexaose and cellopentaose, because further hydrolysis of the reaction can be ignored in this time range used in the present study.

In addition, the reaction products can mutarotate, and this can produce heat [15–17]. As the relaxation constant and the enthalpy change of mutarotation of cellobiose at pH 5.5 and 24.8 °C were reported to be 190 min and -1.8 kJ mol^{-1}, respectively [16], the maximum contribution of mutarotation heat in the condition of the present study should be on the order of 0.2 μW using Eq. (1), assuming the maximum concentration of the product is 1 mM. Considering that the expected contribution based on the published values was very small and that the time course of our observed heat was well explained without considering mutarotation, heat from this source under these conditions, especially in the acidic pH, is negligible. This slow mutarotation of the product also helps to keep our observation simple and clear.

The inhibitory effect exerted by the substrate and/or the reaction products might not be so simple. X-ray crystallography showed that a cellulase can bind to the substrate and/or products in its substrate-binding sites [18]. This clearly indicates that the cello-oligosaccharide becomes the inhibitor as well as the substrate; it seems possible that cellulase in general has such an inhibition mechanism. Further studies, however, are necessary to determine conclusively the mechanisms that govern this inhibitory behavior.

In order to understand or design the cellulase activity and its relationship to the three-dimensional structure of the enzyme, it is necessary to evaluate the enzymatic parameters in an elementary process, such as the hydrolysis of a single glycosidic bond. The results presented in this study clearly show a good example of experimental conditions ideal for observing the heat accompanying the cellulase-catalyzed hydrolysis of a specific glycosidic bond in cello-oligosaccharides. Our previous study on protease activity [6] and the present study on cellulase strongly suggest that the methodology used in this study could be applicable to the easy and precise measurement of many kinds of enzymes.

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