Estimation of Cellulbiohydrolase I Activity by Numerical Differentiation of Dynamic Ultraviolet Spectroscopy

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Abstract 1,4-β-D-glucan cellulbiohydrolase I (CBH I), p-nitrophenyl β-D-cellobioside, p-nitrophenol and cellobiose show distinct ultraviolet spectra, allowing the design of an assay to track the dynamic process of p-nitrophenyl β-D-cellobiose hydrolysis by CBH I. Based on the linear relationship between p-nitrophenol formation in the hydrolysate and its first derivative absorption curve of AUC\textsubscript{340-400 nm} (area under the curve), a new sensitive assay for the determination of CBH I activity was developed. The dynamic parameters of catalysis reaction, such as \( V_m \) and \( k_{cat} \), can all be derived from this result. The influence of β-glucosidase and endoglucanase in crude enzyme sample on the assay was discussed in detail. This approach is useful for accurate determination of the activity of CBHs.

Key words cellulbiohydrolase; dynamic spectrum; area under the curve (AUC)

A fungal cellulase system typically comprises three major classes of enzymes: 1,4-β-D-glucan cellobiohydrolase or exoglucanase (CBH; EC 3.2.1.91); 1,4-β-D-glucan-4-glucan-hydrolases or endoglucanase (EG; EC 3.2.1.4) and β-glucosidase or β-glucoside glucohydrolase (EC 3.2.1.21). The cooperation between CBH and EG is a critical initial step for the hydrolysis of crystalline cellulose. Then the three cellulases cleave β-1,4-glucosidic bonds, with the formation of cello-oligosaccharide and glucose [1]. The heterogeneity and complexity of the cellulase-cellulose reaction makes it difficult to derive a relevant Michaelis-Menten-type rate to express cellulase activity. Because the cellulosic substrates are insoluble, a high concentration of cellulosic sample can not be prepared in an enzyme assay, which is needed for a Michaelis-Menten-type assay. A variety of assay procedures and substrates have been used for measurement of cellulase activities [2,3]. As a major component of the cellulytic system, 1,4-β-D-glucan cellobiohydrolase I (CBH I) has been proposed to play a central role in the biodegradation of natural cellulosics [1]. However, there is no generally accepted method to measure CBH I activity [2].

The water-soluble substrate p-nitrophenyl β-D-cellobioside (PNPC) has been widely used to determine the catalytic activity of CBH [4,5]. At the reaction end of this assay, 10% Na\textsubscript{2}CO\textsubscript{3} must be added to ensure the alkaline environment to enable p-nitrophenol (PNP) to fully develop a yellow color, then the absorbance at 410 nm is used to assess the presence of PNP.

Based on the two basic hypotheses describing the dynamics of enzymatic action, the intermediate complex hypothesis and the steady-state hypothesis, an assay for catalytic enzyme activity requires that the reaction rate be determined by the concentration of enzyme with all the other variables optimized. The essential purpose is to determine the initial reaction rate at the highest substrate concentration (\( K_m \)), because only when the actual initial reaction rate is determined, are \( V \) and \([E]\) linearly related [6-8]. However, those PNPC assays belong to the end-point of titration, and the reaction conditions (such as concentrations of enzyme and PNPC) and the reaction time have to be empirically selected. Using this method, the dynamic properties of catalytic reaction will not be estimated, and no accurate comparison among the outcomes of different experiments is possible.

The dynamic spectrum assay has been effectively
applied in the studies of the structure and function of various biomacromolecules [9–11]. For example, changes in the spectrum of p-nitrophenyl β-D-glucoside have been used to measure the activity of creatine phosphokinase [12]. It is reported here the application of this approach to follow the hydrolysis of PNPC by CBH I, and a new dynamic method for the sensitive determination of CBH I activity is described.

Materials and Methods

Trichoderma pseudokoningii

Trichoderma pseudokoningii S-38 was isolated [13], and CBH I was purified as previously described [14]. Its molecular weight is approximately 66 kDa and the molar extinction coefficient \( \varepsilon = 73,000 \text{ cm}^{-1} \text{M}^{-1} \). PNPC and p-PNP were purchased from Sigma (St. Louis, USA). UV-Vis spectra were measured on a UV-3100 UV-VIS-NIR recording spectrophotometer equipped with a thermostat temperature controller (Shimadzu, Kyoto, Japan) over the range of 200–400 nm using a 1.0 cm path-length quartz cell.

Smooth observed data and statistical analysis

Enzyme assay as a matter of course yields a series of points with an experimental error. Direct numerical differentiation of these points or construction of a model will be greatly influenced by the experimental error. Thus it should be useful for smoothing progress curves before obtaining derivatives [15]. The spline interpolation approach is considered here. Smoothing spline is a popular method for performing nonparametric regression. It approaches a true function subinterval by subinterval [16]. Microsoft Excel, graph software Prism 6.0 (2002) and software TableCurve 2D version 5.0 (http://www.spssscience.com) were used to treat the experimental data. Isosbestic points of derivative absorption curves were used as an index to determine the concentration of PNP formation during hydrolysis of PNPC by CBH I.

An isosbestic point wavelength in the ultraviolet (UV) spectrum indicated the presence of equilibrium between two absorbing species. At this wavelength, the absorbance depended on the total molar concentration of the two absorbing species and not their concentration. Isosbestic points of derivative spectra can be used for quantitative determination of the two absorbing species in equilibrium [17–20].

We showed experimentally an isosbestic point on the UV spectrum curve, which was observed of hydrolysate during PNPC hydrolysis progress by CBH I. Such wavelength changes of isosbestic point were also observed in the mixture solutions consisting of different concentrations of PNPC and PNP. A linear relationship could be established between the PNP concentration and the first derivative curve area in the range of 340–400 nm. It could then be used to calculate the PNP formation during hydrolysis progress of PNPC by CBH I.

For estimation of the total area under curve (AUC) of UV spectra, AUC during hydrolysis to express the effects of time or enzyme concentration, a statistical moment of the Michaelis-Menten elimination kinetic method was used. It is a common nonlinear method used in pharmacokinetics, and the area could be directly calculated by definite integral [21–23]. If concentration values \( C \) are measured at time \( t_i \) \( (i = 1, \cdots, n) \), the numerical integration method might be written as a summation over \( n-1 \) intervals, as shown in Equation 1:

\[
AUC = \int C \, dt = \sum_{i=1}^{n-1} C_{ti} \Delta t
\]

In the present study, the wavelength was used instead of \( t_i \) and absorbance used instead of \( C \), therefore, \( AUC \) is the total intensity of absorbance in the range of \( n \) to \( n-1 \) wavelength.

Results

UV-spectrophotometric properties of CBH I, PNPC, PNP and cellulobiose in acetate buffer

The UV spectra of CBH I, PNPC, PNP and cellulobiose are shown in Fig. 1(A). The absorbance of PNPC was higher than that of PNP between 250 and 305 nm, and it was lower than that between 305 and 400 nm; so one isosbestic point can be observed at 305 nm for PNPC and PNP. And the cellulobiose and CBH I had almost no absorbance beyond the range 240–300 nm. Those spectrophotometric properties could be used to estimate the PNP formation during hydrolysis by CBH I.

The CBH I drives hydrolysis of each PNPC molecule to generate one PNP and one cellulobiose molecule, which can be illustrated as follows:

\[
\text{O} \quad \text{Cellulobiose} \quad | \quad \text{H}_2\text{O} \quad \text{CBH I} \quad 30^\circ \text{C} \quad \rightarrow \quad \text{O} \quad \text{PNP} \quad + \quad \text{Cellulobiose}
\]

The cleavage of the gluconic bond between PNP and
cellobiose generates a distinct spectrum in the 200−400 nm range between the substrate (PNPC) and the product (PNP). Thus it is possible to use spectral patterns to quantitatively determine the amounts of substrate and hydrolysis product at any time point during the progress of the reaction. The effect of cellobiose on the absorbance in the wavelength 250−400 nm was small enough to be ignored.

Comparison of the isosbestic points of PNPC and PNP mixture solution on UV spectra curves in acetate buffer

Although one isosbestic point was observed at 305 nm for PNPC and PNP, the position for each mixture solution depended on the molar ratio of PNPC and PNP (data not shown). Similar phenomena were also observed in the time progress of PNPC hydrolysis by CBH I. Thus, this result used as an index for determination of PNP might lead to errors. This problem could be resolved using its first order derivative curve, as shown in Fig. 1(B,C).

Using this method, it clearly indicated that, for the mixture solutions consisting of PNPC and PNP at different composite ratios, and for the PNPC hydrolysate products hydrolyzed by CBH I at different time points, their isosbestic points were both at 340±1 nm. As described above, AUC, a symmetry peak in the range of 340−400 nm, can be used as an index to express the catalytic activity of CBH I in hydrolysis of PNPC [Fig. 1(D)].
Design of assay conditions to obtain accurate catalysis rate in enzyme assay

The favorable conditions for CBH I catalysis reaction are well known: a pH level of 4.8 and temperature of 50 °C [4,5,7,14]. These conditions were used in present studies. To obtain an accurate record of the reaction progress, the highest substrate concentration (＞K_m) is needed and the conversion rate is within 10% [7,8]. The measurement must be maintained for a considerable period, during which the reaction rate might appear linear [8]. The behaviors of time progress expressed by UV spectra during the hydrolysis of PNPC catalyzed by CBH I was shown in Fig. 1(C) and Fig. 2. It was carried out as follows: 0.8 ml of 250 µM PNPC, 0.1 ml of 1 µM CBH I and 0.1 ml 100 µM sodium acetate buffer, pH 4.8, incubation at 50 °C for more than 20 min; and the dynamic UV spectrum (250–400 nm) was scanned at 5 min intervals.

![Fig. 2](image.png)

**Fig. 2** Time progress of p-nitrophenyl β-D-celllobioside (200 µM) hydrolyzed by 1,4-β-D-glucan cellobiohydrolase I (1.0 µM) at 50 °C in acetate buffer
Expressed by the area under the curve (AUC_240−400 nm) and its first derivative curve.

During the first 10 min of hydrolysis, PNPF formation, as calculated by the increase of AUC_240−400 nm, was linearly correlated with the time of hydrolysis (Fig. 2), however, the virtual linearity of the instantaneous rate of this reaction is within 5 min. Thus, to accurately determine the catalytic rate of PNPC hydrolysis, the reaction time must be within 5 min.

Hydrolysis of PNPC by different concentrations of CBH I

To study the effect of CBH I concentration on the hydrolysis of PNPC, reaction conditions were as follows: 0.05–0.80 µM CBH I (0.1 ml) was mixed with 250 µM PNPC (0.8 ml) and 0.5 M sodium acetate buffer (0.1 ml, pH 4.8), and incubated at 50 °C for 5 min. UV absorbance was measured at 1 min intervals [Fig. 3(A)].

As shown in Fig. 3(B), the virtual linearity between the concentration of CBH I and the catalysis velocities, calculated by the first order derivative of AUC_240−400 nm, was obtained. However, the AUC value shows only relative activity of CBH I. Based on the standard curve of AUC_240−400 nm for PNPF [Fig. 1(D)], CBH I activity shown as PNPF concentration can be easily transformed. Using crude cellulase as the enzyme sample, the value of PNPF formation by a certain volume of crude cellulase can also
Evaluation of the $k_{\text{cat}}$

Evaluation of the $k_{\text{cat}}$ is very important to achieve a detailed analysis of catalysis reaction of an enzyme. It has been defined as “turnover number” or “molecular activity”, which means the number of moles of substrate transformed per minute per mol of enzyme, expressed as $\mu$M product/ml by $\mu$M enzyme per min [6]. The value of $k_{\text{cat}}$ can be obtained through Equation 2:

$$k_{\text{cat}} = \frac{V_{\text{max}}}{[E]}.$$  

However, there are many difficulties in calculating the $V_{\text{max}}$ using the Michaelis-Menten equation [24–27].

In the present study, the $V_{\text{max}}$ and $[E]$, can be directly observed from the plot of instantaneous rate of CBH I activity versus CBH I concentration (Fig. 4) and the smooth data (the insert in Fig. 4). Thus, the $k_{\text{cat}}$ can be easily obtained by simple calculation through Equation 2. Using Equation 2, the $k_{\text{cat}}$ is calculated to be approximately 2756. 3 nM PNP formation per nM CBH I in 1 min at 50 ºC in acetate buffer, pH 4.8. Using this method, it is unnecessary to measure the initial rate to determine $V_{\text{max}}$ and $k_{\text{cat}}$, and they do not depend on the Michaelis-Menten assumption.

Discussion

The widely used PNP assay method [4,5] is unsuitable for the kinetic study, as mentioned above, and the molecular extinction coefficient of PNP is also lower ($\varepsilon_{410\text{ nm}} = 0.0008120 \text{ cm}^{-1}\text{M}^{-1}$), whereas the $\varepsilon$ of PNP expressed as the first derivative AUC$_{340-400\text{ nm}}$ is 5.2 times higher [Fig. 1(D)]. It is suggested that the method of first derivative absorbance curve based on isosbestic points could provide better resolution and higher sensitivity. Many p/o-NPC-like compounds, such as p-nitrophenyl β-D-glucoside, o-NPG(-galactoside) and o-NPF (-fucoside), are widely used in enzyme assay. The method proposed here might also be useful in these fields.

In general, the catalytic rate is calculated by three approaches.

First, using the integrated Michaelis-Menten equation, as shown in Equation 3:

$$\frac{\Delta P}{t} = V_{\text{max}} (1 + \frac{K_{n}}{S}) - \frac{V_{\text{max}}K_{n}}{2(K_{n} + S)} \Delta P$$  

by this method, the intercept at $P_0$ of the resulting curve is necessarily $dP/dt$ at $t=t_0$, which is the initial rate [21]. Because of the complexities of the integrated rate equation, this method has not gained wide acceptance.

Second, the initial rate is defined as the reaction rate at the early phase of enzymatic catalysis, which equals the rate near the beginning of the reaction. However, the slope at the origin of the progress curve has the highest change that is even more unreliable than that expected, and lacks predicative power for the entire reaction mechanism [16, 17]. In practice, the catalytic rate is measured in certain interval at a fixed temperature as other factors are maintained, then calculated using some kinetic equations. Under this condition, the obtained catalysis rate might depend on the timescale and temperature range used in the measurement and the selected equations. In fact, most of the experimental data lie in the range of the slower reaction period. For many enzymes, the progress curves monitored by product or substrate consumption and enzyme inactivation are non-linear. The kinetic behavior of enzymes can be described in terms of a hyperbolic or sigmoid relationship between measured response and controlled variables [24]. Under these conditions, estimation of the initial rate based on the Michaelis-Menten assumption is a subjective and inexact progress [25–27].

Finally, the catalytic rate continuously changes during reaction progress based on the chemical kinetics. Mathematically, those changes can be expressed as its instantaneous rate and can be easily evaluated by the change in the slope of a tangent line at any point on the reaction curve, which is not affected by the curve’s shape and only depends on its position on the curve [28–30].

Waley [31] proposed an easy method for the determina-
nation of the initial rate that is based on the differentiation rule. The rate near the beginning of an enzyme-catalyzed reaction can be found accurately from the slope of a chord joining two points on the progress curve. The instantaneous rate of enzyme reaction can be found accurately from the slope of a chord joining two points on the progress curve [26–30], which can be shown as Equation 4:

\[
-\frac{dC}{dt}_{\text{max}} = k
\]

where \(C\) is the concentration of substrate, \(t\) is the time, and \(k\) is the reaction constant. This approach has been generally used and further developed to the kinetic analysis of the entire reaction progress rather than the initial rate [24,32,33].

Some modifications are made on those previous studies in our study: (1) smoothing experimental data by the spline interpolation method for permitting the determination of the instantaneous rate; (2) using the isosbestic point of derivative absorbance curves as an index to determine the concentration of PNP; (3) evaluating a suitable reaction period, in which a linear relationship would be established between enzyme concentration and catalytic activity, according to the plot of first derivative AUC 340/400 nm versus concentrations of CBH I; and (4) using PNPC at a higher concentration as the substrate, which was hydrolyzed by a series of CBH I or crude cellulase sample solutions at different concentrations. The \(V_{\text{max}}\) and \([E]\) can be directly observed from the plot of the instantaneous rate of CBH I activity versus the concentration of CBH I. Then \(k_{\text{cat}}\) can be obtained by simple calculation.

Our assay procedures were quite good not only for pure CBH I preparation but also for crude cellulase preparation, as we take into account any interference from EG and \(\beta\)-glucosidases [5]. As EG has no catalysis capacity for PNPC [2,4,5], the possibility of influence from it is ruled out. Also, although PNPC, in some degree, can be hydrolyzed by \(\beta\)-glucosidases, its content in crude cellulase is much lower than that in CBHs. For typical cellulolytic fungi, such as Trichoderma spp, Penicillium spp and Phanerochaete chrysosporium, the content of \(\beta\)-glucosidases and little CBHs, hence it is a kind of non-typical cellulolytic fungus [30].

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References

17. Berlett BS, Levine RL, Stadtman ER. Use of isosbestic point wavelength shifts to estimate the fraction of a precursor that is converted to a given product. Anal Biochem 2000, 287: 329–333
18. Wahbi AA, Hassan EM, Gazy AA. Use of isosbestic points of derivative
21 Perrier MGD. Pharmacokinetics. 2nd ed. New York: Marcel Dekker 1982
22 Rescigno A. Area under the curve and bio-availability. Pharma Res 2000, 42: 539–540
26 Huang C. Derivation and initial velocity and isotope exchange rate equation.

Methods Enzymol 1979, 63: 54–84
27 Allison PD, Purich DL. Practical considerations in the design of initial velocity enzyme rate assay. Methods Enzymol 1979, 63: 3–22
30 Wahl RC. The calculation of initial velocity from product progress curves when [s]<[Km]. Anal Biochem 1994, 219; 383–384
33 Koerber SC, Fink AL. The analysis of enzyme progress curves by numerical differentiation including competitive product inhibition and enzyme reaction. Anal Biochem 1987, 165: 75–87
34 Klyosov AA. Trends in biochemistry and enzymology of cellulose degradation. Biochemistry 1990, 29: 10577–10585

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