Combined Product and Substrate Inhibition
Equation for Cellobiase

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Summary
Cellobiase (EC 3.2.1.21) is a β-glucosidase which hydrolyzes cellobiose to glucose and is known to be subject to both product and substrate inhibition. This work reports a model which combines both product and substrate inhibition effects for cellobiase isolated from a commercial preparation of Trichoderma viride from Miles Laboratories (Elkhart, IN). An integrated rate equation is presented which predicts the trends of time courses for hydrolyses of cellobiose at concentrations ranging from 14.6–146mM cellobiase. The constants used in the model (determined from initial rate data) are compared to those reported for cellobiase obtained from other sources of T. viride. Most notable in this comparison is the apparently higher activity and reduced inhibition of this enzyme compared to other sources of cellobiase.

INTRODUCTION
Cellobiase (EC 3.2.1.21) is a β-glucosidase which hydrolyzes cellobiose to glucose and is subject to product\(^{-1}\) and/or substrate\(^{-2}\) inhibition. This article reports a kinetic equation which combines substrate and product inhibition effects for a cellobiase isolated from a commercial cellulase preparation from Miles Laboratories. Curve fitting to initial rates and initial rate patterns from a Lineweaver–Burk plot were used to postulate the initial rate equation and to determine values of the Michaelis and inhibition constants. The integrated form of this equation is shown to follow the trend of time-course hydrolysis data obtained for cellobiase with respect to cellobiose.

MATERIALS AND METHODS

Reagents

Cellulose (Sigma Chemical Co., St. Louis, MO) and glucose (Fisher Scientific, Fair Lawn, NJ) were used to study enzyme kinetics. Bovine serum albumin (Sigma) was used as a standard for protein determinations by the methods of Lowry et al.

Enzyme

A commercial preparation of cellulases from Trichoderma viride from Miles Laboratories (product No. 454500, Elkhart, IN) served as starting enzyme from which cellulose was isolated. Crude cellulase powder was added to 10mM sodium phosphate buffer (pH 6.8) containing 0.2mM EDTA to give a 10% (by wt.) suspension. Insoluble materials were spun down at 4°C and 2 × 10^5 g. The supernatant was applied to 1.5 × 24 cm DE-52 (Whatman Co., Clifton, NJ) DEAE-cellulose column. Sodium acetate, 50mM (pH 5.8), was passed through the column to wash out extraneous proteins. A linear salt gradient of from 50–200 mM sodium acetate (pH 5.8) then eluted the cellulase from the column. Fractions containing cellulase activity were pooled and concentrated by ammonium sulfate precipitation. The concentrated cellulase (volume 0.5 mL) was then placed on a Sephadex G-150, 1.5 × 120-cm column (previously equilibrated with 50mM sodium acetate buffer, pH 5.8), and eluted with 50mM sodium acetate. The fractions showing cellulase activity were again pooled and then concentrated in a Diaflo ultrafiltration cell with a PM 10 membrane (Amicon, Lexington, MA).

Enzyme Stability

The stability of cellulase at 50°C and 100mM sodium acetate buffer at pH 4.8 was checked over a 60-day time period (Fig. 1). The loss of activity in one hour is insignificant; 35% of the activity is lost in 60 days.

![Graph](image)

Fig. 1. Deactivation of cellulase at 50°C. Cellulase was kept in 100mM sodium acetate buffer (pH 4.8). Aliquots were withdrawn and activity was measured with respect to 14.6mM cellulose. Relative activity of 1 corresponds to a rate of 0.29mM glucose min⁻¹ with Δ₀ = 1.83 μg/mL.
Enzyme Kinetic Assay

Enzyme activity was determined by measuring glucose appearance with a Beckman (Fullerton, CA) glucose analyzer and/or cellulobiose disappearance by liquid chromatography (LC). In an initial rate assay, a 2-mL volume having a composition of 1.83 μg/mL enzyme protein 100 mM sodium acetate buffer (pH 4.8), 0.05% sodium azide (to prevent bacterial growth), and from 0.5-300 mM cellulobiose was incubated at 50°C for 20 min. The mixture was then placed in a boiling-water bath for 5 min to inactivate the enzyme, followed by standing at ambient conditions for 30 min to bring the incubation mixture to a temperature compatible with the requirements of the glucose analyzer. The assay mixture was then analyzed. The pH (4.8) and temperature (50°C) utilized are optimum for this enzyme.

Product inhibition was examined by adding glucose to the assay mixture to give an initial glucose concentration of 5.5 or 11.1 mM. All other conditions were the same.

RESULTS

The initial rate data plotted in a Lineweaver-Burk plot [Fig. 2(a)] and replots (Fig. 3) show that cellulobiose is noncompetitively inhibited by the product glucose. Kinetic constants (K_m, K_i1, K_i2, and V_max) calculated using previously described methods are given in Table 1.

While substrate inhibition is not apparent for cellulobiose concentrations below 10 mM [Fig. 2(a)], it becomes noticeable at 15 mM cellulobiose and

<table>
<thead>
<tr>
<th>TABLE 1 Values of Cellulobiose Kinetic Constants</th>
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<tbody>
<tr>
<td>Constant</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>K_m (mM)</td>
</tr>
<tr>
<td>K_i1 (mM)</td>
</tr>
<tr>
<td>K_i2 (mM)</td>
</tr>
<tr>
<td>K_i (mM)</td>
</tr>
<tr>
<td>V_max (μmol glucose/ min/mg protein)</td>
</tr>
<tr>
<td>V_i (μmol glucose/ min/mg protein)</td>
</tr>
<tr>
<td>k_1 (μM glucose/ min mg protein/mL)</td>
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<tr>
<td>k_2 (μM glucose/ min mg protein/mL)</td>
</tr>
</tbody>
</table>

* Also refer to eqs. (2)-(5).
Fig. 2. Lineweaver-Burk plots for cellulose: (a) showing noncompetitive product inhibition with initial glucose concentration as indicated; and (b) showing substrate inhibition at high substrate concentration. Each data point is an average of results from four initial rate assays. $K_s = 1.83 \mu M$.

Fig. 3. Replots of (a) slope and (b) intercepts from Fig. 2(a) as a function of glucose concentration.
INHIBITION OF CELLOBIOASE

The observed substrate inhibition suggests that one of the additional reaction sequences,

$$EC + C \xrightarrow{k_1} CEC \quad \text{(1)}$$

or

$$EC + C \xrightarrow{k_0} CEC \xrightarrow{k_0} EC + 2G \quad \text{(2)}$$

should be added to the model for noncompetitive product inhibition$^{14}$:

$$E + C \xrightarrow{k_1} EC \xrightarrow{k_0} E + 2G \quad \text{(3)}$$

$$E + G \xrightarrow{k_0} EG \quad \text{(4)}$$

$$EC + G \xrightarrow{k_0} ECG \quad \text{(5)}$$

A reaction step for reversion is not included since cellubioase reaction mixtures high in glucose analyzed by this LC method$^{13}$ (which is capable of separating cellobextrins and detecting concentrations at least as low as 0.1 mM) show only glucose after 12 h. Solutions initially containing 0.88 mM cellubioase and 53.9 mM glucose, 1.46 mM cellubioase and 52.7 mM glucose, and 2.05 mM cellubioase and 51.6 mM glucose, respectively, were examined at $E_0 = 1.83 \mu g/mL$. From these results, the magnitude of reverse reaction was assumed to be small relative to the forward reaction and hence, the possibility of oligosaccharide formation was not included in eqs. (1) and (2). Using the pseudo-steady-state assumption and conservation of enzyme, eq. (6) [for reaction sequence eqs. (1), (3), (4), and (5)]:

$$\frac{dG}{dt} = \frac{V_{\text{max}}}{(K_m/C)[1 + (G/K_{i1})] + [1 + (G/K_{i2})] + C/K_s} \quad \text{(6)}$$

and eq. (7) [for reaction sequence eqs. (2), (3), (4), and (5)]:

$$\frac{dG}{dt} = \frac{V_{\text{max}} + V_1(C/K_s)}{(K_m/C)[1 + (G/K_{i1})] + [1 + (G/K_{i2})] + C/K_s} \quad \text{(7)}$$

are obtained. The constants are defined in Table I.

The rate equation best describing the substrate inhibition was chosen by fitting the initial rate data to both eqs. (6) and (7) by least squares. The terms $G/K_{i1}$ and $G/K_{i2}$ are small for initial rate studies when no
glucose is added, and hence eqs. (6) and (7) used for fitting simplify to

\[
\frac{dG}{dt} = \frac{V_{\text{max}}}{(K_m/C) + 1 + (C/K_s)}
\]

(8)

\[
\frac{dG}{dt} = \frac{V_{\text{max}} + V'(C/K_s)}{(K_m/C) + 1 + (C/K_s)}
\]

(9)

The values of \(K_m\) and \(V_{\text{max}}\) are given in Table I.

Figure 4 gives the best fit lines for eqs. (8) and (9), respectively. Equation (9) gave the better fit. On this basis, eq. (2) was chosen as best representing the substrate inhibition reaction and eq. (7) as giving the initial rate expression for combined substrate inhibition and noncompetitive product inhibition. Values for \(K_s\) and \(V'/E_0\) obtained from the fit of eq. (9) are 40.5 mM cellobiose and 45.5 \(\mu\)mol glucose \(\text{min}^{-1} \cdot \text{mg enzyme}^{-1}\) \((V' = 83 \mu \text{g glucose/min at } E_0 = 1.83 \mu \text{g/mL})\), respectively.

The substrate concentration giving the maximum initial rate is independent of enzyme concentration and can be computed from the equation

\[
C_0 = \frac{V'K_m + \sqrt{(V'K_m)^2 - V_{\text{max}}K_mK_s(V' - V_{\text{max}})}}{V_{\text{max}} - V'}
\]

(10)

Using values of the kinetic constants from Table I, the cellobiose concentration giving the maximum rate of 158 \(\mu\)mol glucose \(\text{min}^{-1} \cdot \text{mg protein}^{-1}\) \((289 \mu \text{g glucose/min at } E_0 = 1.83 \mu \text{g/mL})\) is 18.1 mM.

The equation for predicting the cellobiose hydrolysis time course is obtained by rearranging eq. (7) and integrating

\[
\int_0^t \frac{dG}{\left[\left(\frac{K_m}{C} + 1 + (G/K_s)\right) + \left(\frac{G}{K_s} + 1 + \frac{C}{K_s}\right)\right]} \frac{V_{\text{max}} + V'(C/K_s)}{V_{\text{max}} + V'}
\]

(11)
on a mole basis, $C = C_0 - \frac{R}{G}$, and the integrated form of eq. (11) is

$$0.5T = \frac{K_r}{V} \left[ 1 + \left( \frac{V_{\text{max}}}{V} \right) \left( \frac{K_s}{0.5K_{i,1}} - 1 \right) - \frac{K_m}{0.5K_{i,2}} \right. $$

$$+ \left. C_0 \left( \frac{1}{0.5K_{i,1}} - \frac{K_m}{0.5K_{i,2}V_{\text{max}}} \right) - \frac{K_m}{V_{\text{max}}} \right]$$

$$\times \ln \left( \frac{V_{\text{max}} + (V/V_s)C_0}{C_0 + 0.5G} \right)$$

Equation (12), together with the values of the kinetic constants (see Table I) gave the predicted time-course curves in Figure 5. The calculated curves give an accurate picture of the trend in the data. However, above 50% conversion the time course is overpredicted.

The curves represent the calculated projection of the model and rate constants determined from initial rate data taken during the first 20 min.

![Graph showing time-course curves](image_url)

Fig. 5. Comparison of cellulase hydrolysis time-course data to predicted time-course curves. Hydrolysis conditions: 50°C, pH 4.8, KCl = 1.6 mM, and Ce = 0.92 mg/mL. C2 indicates initial cellulase concentration. Hydrolysis carried out in sealed vial to minimize evaporation. Initial volume is 20 mL.
<table>
<thead>
<tr>
<th>Source of Cellulose</th>
<th>Miles</th>
<th>Enzero*</th>
<th>Onozuka &quot;SS&quot; **</th>
<th>T. viride HQM 9414***</th>
<th>T. viride† (Worthington Co.1)</th>
<th>T. viride‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature of enzyme assay (°C)</td>
<td>50</td>
<td>50</td>
<td>40</td>
<td>50</td>
<td>25</td>
<td>Not given</td>
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<tr>
<td>V_{max} (mM glucose)</td>
<td>235</td>
<td>116</td>
<td>33</td>
<td>—</td>
<td>—</td>
<td>97 ± 20</td>
</tr>
<tr>
<td>E_{max} (mg protein)</td>
<td></td>
<td></td>
<td></td>
<td>—</td>
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</tr>
<tr>
<td>K_{m,1} (mM)</td>
<td>5.6</td>
<td>2.5</td>
<td>1.5</td>
<td>1.5</td>
<td>2.68</td>
<td>1.8 ± 0.086</td>
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<tr>
<td>K_{e,1} (mM)</td>
<td>22.6</td>
<td>16.4</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>K_{c,1} (mM)</td>
<td>28.3</td>
<td>1.22</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>K_{s,1} (mM)</td>
<td>40.9</td>
<td>—</td>
<td>Substrate inhibition noted</td>
<td>Substrate inhibition noted</td>
<td>No substrate inhibition found up to 34mM cellulose</td>
<td>31.5 ± 7.0</td>
</tr>
</tbody>
</table>

* Reference 4.
** Reference 10.
*** Reference 8.
† Reference 1.
‡ Data from summary Table V in ref. 9.

* Glucose was found to be an activator in the concentration range of 4–85μM, an inhibitor at levels above 8.5mM glucose, and a total inhibitor at 102mM glucose.
of the reaction to a time course extending over 60 h. Hence, a cause for the deviation above 50% may be the presence of smaller deviations at low conversions which accumulate as the predicted time course is extended over a longer range.

**DISCUSSION**

Cellobiase isolated from the Miles commercial cellulase preparation is subject to both substrate and product inhibition. The kinetic constants for this enzyme, however, differ from constants reported for cellobiase isolated from other sources of *Trichoderma viride*. Previously reported values of maximum velocity, $V_{max}$, and Michaelis constant, $K_m$, and product inhibition constants for this enzyme indicate that it is not sensitive to glucose inhibition as other cellobiases. In comparison, the constant reflecting substrate inhibition is similar to that reported by Brown and his co-workers. The cellobiase in the Miles enzyme appears to be less subject to product inhibition than the other enzymes and in this regard may offer an improvement over the other enzymes. While growth conditions for the Miles enzyme are not published, the kinetic data may indicate that cellobiase characteristics may vary depending on growth conditions.

Equation (12) combines both noncompetitive product inhibition and substrate inhibition. While a less complex empirical model could also have been used to curve-fit the data, the approach reported in this communication directly reflects the initial rate data and provides an extrapolation to high conversions on a more objective basis than an empirical model. Consequently, this formulation of the kinetics of this enzyme may prove useful for predicting trends in the time course of hydrolysis at high cellobiase concentrations.

**Nomenclature**

C  cellobiase
E  enzyme (cellobiase)
EC enzyme-cellobiase complex
ECG enzyme-cellobiase-glucose complex
EG enzyme-glucose complex
CEC cellobiase-enzyme-cellobiase complex
G  glucose
$k_i$ kinetic constant
$K_{i,1}$ dissociation constant for $ECG$ complex
$K_{i,2}$ dissociation constant for $EG$ complex
$K_m$ Michaelis constant
$K_i^{'}$ dissociation constant for $CEC$ complex
$K_e$ Michaelis constant as indicated by eq. (2)
$t$ time
References


Accepted for Publication April 28, 1981