Cellulose Hydrolysis by Endoglucanase (Glucan Glucanohydrolase) from Trichoderma reesei: Kinetics and Mechanism

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Summary

Glucanohydrolase from Trichoderma reesei, having a molecular weight of 32,000, was evaluated for kinetic properties with respect to cellulose. Results from this work include: 1) initial rate studies that show that glucanohydrolase hydrolyzes cellulose by a competitive mechanism and that the product, glucose, inhibits the enzyme; 2) low-pressure aqueous liquid chromatography that shows that formation of a reversion product, cellotriose, is minor and occurs in detectable amounts only at very high (90mM) cellulose concentrations; 3) development of an equation based on the mechanism of glucanohydrolase action as determined by initial rate kinetics, which accurately predicts the time course of cellulose hydrolysis; 4) derivation of an initial rate expression for the combined activity of cellulase and glucanohydrolase on cellulose. Based on data in this paper it is shown that the difference in inhibition patterns of the two enzymes could be used for determining the contamination of one enzyme by small quantities of the other.

INTRODUCTION

The cellulase component 1,4-β-glucan glucanohydrolase is an enzyme that has activity with respect to cellotriose and higher cellobiose, swollen cellulose, amorphous cellulose, and filter paper.1-4 It is also reported to have a small activity with respect to cellobiose, although it has not previously been known whether this activity is inherent to the enzyme or due to a slight contamination by cellulase. Recently it has been reported that glucose production from the hydrolysis of cellulose in the absence of cellulase (i.e., β-glucosidase) might be attributed to either the combined action of exo- and endoglucanases, or the presence of some other route.5 The data of Hsu et al.6 clearly show that 1,4-β-glucan cellulohydrolase (i.e., exoglucanase) has no activity with respect to cellobiose. Glucanohydrolase (i.e., endoglucanase) does have activity with respect

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to cellobiose and hence accounts for the appearance of glucose even when no cellobiose is present.

This paper presents a kinetic model for glucan glucanohydrolase from *Trichoderma reesei* (formerly *Trichoderma viride*). The model was postulated based on kinetic patterns obtained from initial rate patterns. Kinetic constants calculated from initial rate data used together with the integrated rate equation derived from the model gave good results in predicting the time course of cellobiose hydrolysis by glucanohydrolase at two substrate concentrations (11.7 and 38.8mM) at conversions of up to 90%. This work indicates that glucanohydrolase from *T. reesei* has a much lower activity toward cellobiose than does cellobiose, and that the mechanism of cellobiose hydrolysis is different for glucanohydrolase and cellobiose.

**MATERIALS AND METHODS**

**Enzyme**

Glucan glucanohydrolase was purified from a cellobiose preparation of *T. reesei* (formerly *T. viride*). Purification, characterization, and confirmation of homogeneity of this enzyme were performed as described in a prior publication. The techniques used for the preparation of crude extracellular cellulase from *T. reesei* (Enzyme Development Corp., New York, NY) were identical to those described previously. The procedures for purification of glucanohydrolase from crude cellulase also were similar to those reported earlier. The crude cellulase from the commercial preparation was first applied to a DEAE-Sephrose column (Pharmacia, 1.5 x 25 cm). Cellobiose and low-molecular-weight endocellulases were eluted from the column with water. Following thorough washing of the column by water, high-molecular-weight glucanohydrolase was eluted by a linear salt gradient of sodium phosphate. The protein fractions containing glucano-hydrolase activity were pooled, concentrated, and the salts removed by gel filtration with a Sephadex G-25 column.

The purity of the enzyme was analyzed by gel filtration on a Sephadex G-100 column as well as by SDS-polyacrylamide gel electrophoresis. Protein was determined by the method of Lowry et al. using bovine serum albumin (BSA) as a standard.

Another test used was immunodiffusion. Antisera against glucanohydrolase were prepared as follows: A 0.5 ml solution containing about 1 mg purified enzyme was emulsified with an equal volume
of complete Freund adjuvant and injected into a rabbit. Injections were repeated four times over a four-week interval, and blood was collected one week after the last injection. Antiserum was prepared by the conventional method.

Double-diffusion tests were carried out on 1% (w/v) agarose gels containing 0.15M NaCl, 0.01M sodium phosphate buffer (pH 6.8), and 0.02% NaN₃. The reaction was allowed to take place overnight in the cold.

Analytical

The hydrolysis rate of cellobiose was measured by monitoring the appearance of glucose using a commercially available glucose analyzer (Beckman Company, Fullerton, CA). In a typical enzyme assay appropriate quantities of cellobiose, enzyme, and buffer (pH 4.8) were combined to give 0.7 ml and incubated at 40°C. The glucose formed was measured on the Glucose Analyzer. Assay conditions were chosen so that glucose formation was linear with respect to both time and enzyme concentration. All results were corrected against a reagent blank.

An international unit (IU) is defined as the µmol glucose formed/min at the conditions of the assay.

Formation of higher-molecular-weight cellooligos was checked using a new form of liquid chromatography known as low-pressure liquid chromatography (LPLC). In this type of chromatography, carbohydrates are separated on an ion-exchange resin using water as the sole eluant. The eluting peaks from the column were quantitated by a refractive index detector connected to an integrator which automatically integrated the area under the peaks.

Aqueous LPLC has several advantages over reverse-phase chromatography (RPC) in the analysis of cellooligos. Carbohydrate samples containing (buffer) salts and (enzyme) protein can be injected “as is” into an aqueous LPLC, whereas these samples must be “cleaned-up” before injecting into a RPC system. The reason for this is that the partially aqueous organic eluent (usually acetonitrile) used in RPC tends to precipitate out proteins and salts, hence, adversely affects the instrument operation. Furthermore, partially aqueous solutions also tend to precipitate, at least partially, higher-molecular-weight cellooligos that are otherwise soluble in a totally aqueous solvent. Hence, aqueous LPLC is the preferable, method for cellooligos analysis where precise and reliable results are needed for enzyme kinetic studies. Unlike RPC, the procedures and eluent used with LPLC ensure maintenance of the
sample integrity throughout the assay procedure. An added advantage of LPLC is that it has 50 times more sensitivity than the currently available commercial RPC systems. Together, these factors have prompted considerable efforts in our laboratory to develop aqueous LPLC for assay and quantitation of the activity of the cellulases with respect to the enzyme's true substrates, i.e., cellulose and cellobiose.

In a typical enzyme assay using LPLC, appropriate quantities of cellobiose or cellobiose, enzyme, and buffer (pH 4.8) were combined to give 0.7 to 1 ml total volume and incubated at 40°C. At the end of the incubation period, a 20 µl sample of the mixture was taken and directly injected into the LC.

**Optimum pH and Temperature**

The optimum pH was determined using cellobiose as a substrate. Cellobiose was chosen since glucanohydrolase is known to react rapidly with cellobiose and higher oligosaccharides while having a relatively low affinity for cellobiose. Thus, the optimum pH was obtained for enzyme activity with respect to a substrate for which the enzyme has high activity. This is anticipated to reflect the condition at which the enzyme would most likely be used in a practical situation. The cellobiose for these experiments was prepared by aqueous, preparative scale, low-pressure chromatography as described elsewhere. To measure the optimum pH, the enzyme was incubated at 40°C in a mixture that contained 7mM cellobiose and 2 to 4mM sodium acetate buffer. The pH of the buffer ranged from 3 to 7. Enzyme activity was computed from the initial rate of appearance of cellobiose which was measured by LPLC (see Fig. 1 for sample chromatograms).

The optimum temperature was determined by incubating the enzyme at either 40 to 50°C for various lengths of time and then assaying for residual activity at 40°C, again using cellobiose as substrate.

**Kinetic Study**

Using the enzyme assay procedure described above, initial rates of cellobiose hydrolysis were measured at substrate concentrations ranging from 1 to 10mM. In a typical assay, enzyme, sodium acetate
buffer (pH 4.8), and cellulose were combined to give a total volume of 0.7 ml and incubated at 40°C for 30 to 90 min. At the end of the incubation period the glucose formed was measured by injecting a 10 to 50 µl sample of the mixture into the Beckman glucose analyzer. When small quantities of glucose were added to the initial incubation mixtures, inhibition of the cellulose hydrolysis reaction occurred. Hence, a series of initial rate studies were performed where both initial cellulose and glucose concentrations were varied.

The data, consisting of measured initial rates of glucose appearance as a function of substrate concentration, were plotted in the form of inverse rate versus inverse substrate concentration. The use of this plot together with the appropriate derivation of Michaelis-Menten kinetics allows development of a kinetic model of enzyme action.

RESULTS

Physical Characteristics of Purified Glucanohydrolase

Total glucanohydrolase protein was less than 8% of the total high-molecular-weight soluble protein. Two fractions of glucohydro-
lases were isolated, a high-molecular-weight (52,000) enzyme which was
the major component and a low-molecular-weight (18,000) fraction which
was the minor component. Kinetic studies were done using the major
(high-molecular-weight) component.

The purity of the enzyme preparation was verified by SDS–poly-
arylamide gel electrophoresis.25 The purity of glucanohydrolase
obtained after DEAE–Sepharose column chromatography was ver-
fied by SDS-polyacrylamide gel electrophoresis (Fig. 2) as well as
by gel filtration of enzyme on a Sephadex G-100 column (Fig. 3).
As can be seen (Fig. 2) the purified enzyme has no detectable
contaminants.

The antisera against purified glucanohydrolase, used to test the
cross-contamination by cellulase and cellobiohydrolase by the im-
munodiffusion assay, showed no cross-reactions between glucano-
hydrolase–cellobiase within the inherent resolution of this technique.
This indicates that the purified glucanohydrolase was free from
cellobiase contamination. Moreover, there was no cross-reaction
between glucanohydrolase and cellobiohydrolase. This indicates
that the glucanohydrolase was free from cellobiohydrolase contam-
ination as well.

Fig. 2. SDS–polyacrylamide gel electrophoresis of purified glucanohydrolase.
Enzyme was chromatographed on a 10 cm 7.5% polyacrylamide gel with 0.1% SDS
as described previously.25 Graph shows the scan of such a gel after staining with
Coomassie brilliant blue.
Fig. 3. Sephadex G-100 column (1.5 x 90 cm) chromatography of glucanohydrolase. Enzyme obtained after DEAE-Sepharose chromatography was pooled and concentrated and applied to the column. Column was eluted with 0.05M sodium acetate (pH 4.8); 6 ml fraction were collected. (○) Absorbance at A_{280}; (●) glucanohydrolase activity at reducing sugar released from CMC, absorbancy at A_{490}.

Optimum pH and Temperature

The pH profile for glucanohydrolase (Fig. 4) shows the optimum pH to be 4.8. The lack of reversion products (Fig. 1) indicates that pH has little, if any, effect on the formation of these products.

The optimum temperature for kinetic studies is 40°C. As shown in Figure 5, the enzyme rapidly loses activity at 50°C while it is very stable at 40°C. At 50°C the enzyme half-life was on the order of 1 hr. At 40°C the enzyme activity was found to be stable for at least one day.

All kinetic data reported in this paper were obtained from enzyme assays performed at 40°C and pH 4.8.

Kinetic Study

Data for glucanohydrolase activity with respect to cellobiose hydrolysis are plotted in Figure 6. The straight line (open circles, no glucose added) shown in Figure 6(a) resulted. This line simply indicates that as substrate concentration increases, the rate also increases. When small amounts of glucose are added and initial rate studies are repeated, the lines indicated by the squares and triangles result. In these cases, due to the inhibitory effect of glucose, the
rate is smaller at a given substrate concentration when glucose is initially added. The resulting pattern of lines, giving what is known as a Lineweaver-Burk plot, indicated that the enzyme is product inhibited by a competitive mechanism. (See Appendix in Ref. 10 for explanation of types of inhibition indicated by Lineweaver-Burk plots.)
Product Inhibition

The pattern of lines in Figure 6 intersecting on the y axis suggests competitive product inhibition for which the Michaelis-Menten equation is

$$v = \frac{V(GG)}{K(1 + G/K_{1,2}) + GG}$$  \hspace{1cm} (1)

A detailed derivation is given in the Appendix. At zero inhibitor (i.e., glucose, $G = 0$), eq. (1) reduces to

$$v = \frac{V(GG)}{K + GG}$$  \hspace{1cm} (2)

which is the standard form of the Michaelis-Menten equation.

The inverted form of eq. (1):

$$\frac{1}{v} = \frac{K}{V} \left(1 + \frac{G}{K_{1,2}}\right) \left(\frac{1}{GG}\right) + \frac{1}{V}$$  \hspace{1cm} (3)
is of the form

\[ y = mx + b \]  \hspace{1cm} (4)

i.e., the form of a straight line. Hence, eq. (4) predicts that a Lineweaver-Burk plot of the data should be linear. A replot of the slope

\[ (K/V)(1 + G/K_{1a}) \]

as a function of \( G \) should also be linear if the reaction sequence reflects competitive inhibition. By these criteria, then, Figure 6 indicates that the mechanism is consistent with the data.

Since the plot and replot (see Fig. 6) are linear, values of the kinetic constants \( K \) and \( K_{1a} \) can be determined directly from them. The \( x \) intercept of the replot gives \(-K_{1a}\). The \( x \) intercept of the Lineweaver-Burk plot gives \( K \) and the \( y \) intercept, \( E_{\text{in}}/V \). The values of these kinetic constants, obtained from lines fit to the data in Figure 6 by linear least squares, are summarized in Table I. Note that the reaction velocity is shown in terms of specific activity. This is equivalent to multiplying eq. (1) through by the constant \( 1/E_{\text{in}} \).

The mechanism of action on cellulose by glucanohydrolase is competitive as is shown by the initial rate data. Thus, integration of the rate expression [eq. (3)] should give an equation that will accurately predict the hydrolysis time course of cellulose. Recalling that \( v = dG/dt \), eq. (1) may be rearranged to give:

\[ V \int_{n}^{t} dt = \int_{n}^{G} \left[ K \left( 1 + \frac{G}{K_{1a}} \right) + GG \right] \frac{dG}{GG} \]  \hspace{1cm} (5)

where

\[ GG = (GG_{n}) - G/2 \]  \hspace{1cm} (6)

Substitution of eq. (6) into eq. (5) and integration results in:

\[ Vt = \left( 1 - \frac{2K}{K_{1a}} \right) (G) + K \left( 2 + \frac{4(GG_{n})}{K_{1a}} \right) \ln \left( \frac{GG_{n}}{GG_{n} - G/2} \right) \]  \hspace{1cm} (7)

TABLE I
Summary of Kinetic Constants for Glucanohydrolase

<table>
<thead>
<tr>
<th>Constant</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( V/E_{\text{initial glucose-min mg protein}} )</td>
<td>0.58</td>
</tr>
<tr>
<td>( K_{\text{cat}} \text{cellulose} )</td>
<td>1.6</td>
</tr>
<tr>
<td>( K_{1a} \text{cellulose} )</td>
<td>0.98</td>
</tr>
</tbody>
</table>
Given values of the kinetic constants \( V, K_s, \) and \( K_{i,5} \) and the initial substrate concentration \( [GG]_0 \), the time \( t \) required to attain a certain product concentration \( G \) may be calculated from eq. (7).

A time-course study at two starting concentrations of cellobiose (5.85 and 38.8 mM) showed eq. (7) to accurately predict the rate of hydrolysis of cellobiose (Fig. 7). The experiment was carried out at pH 4.8, 40°C, and with reaction times not exceeding 25 hr. The predicted curves (solid lines in Fig. 7), computed from eq. (7) using the values of kinetic parameters determined from the initial rate study agree with the experiment data (squares in Fig. 7).

If all the product were removed as it was formed, there would be no inhibition. The time course of the reaction for such a situation

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Fig. 7. Time course of hydrolysis of cellobiose by glucanohydrolase. (—) Data predicted by competitive inhibition model. (---) predicted for case of no glucose inhibition. (a) \( [GG]_0 = 38.8 \text{ mM}; E_{ac} = 0.118 \text{ mg/mL}; \text{temperature} = 40°C \). (b) \( [GG]_0 = 5.85 \text{ mM}; E = 0.0672 \text{ mg/mL}; \text{temperature} = 40°C \).
can be predicted by using eq. (1) where \( G = 0 \) and integrating:
\[
V_t = (G_2 \cdot G - 2K \ln[1 - G/2(GG_3)])
\]

(8)

Computation using eq. (8) shows that the reaction would proceed much faster if the product were removed as it was formed (indicated by dotted lines in Fig. 7).

The competitive pattern of glucanohydrolase action (Fig. 6) implies that the noncompetitive reaction step:
\[
E^* + G = E^*G
\]

(9)
is at most very small. Low-pressure liquid chromatography analysis (Fig. 8) of the hydrolysate done at the end of the run shown in Figure 7(b), shows no detectable cellotriose (\( G_3 \)). This is further indication that the reaction given by eq. (9) is small since \( G_3 \) formation would require the presence of the enzyme complex \( E^*G \).

At high enough cellotriose concentrations, however, some \( G_3 \) is formed. The chromatograms in Figure 9 resulted from incubation of glucanohydrolase with approximately 90mM cellotriose. At time zero there is essentially pure cellodextrin (Fig. 9(a)). After 72 min, a small amount of \( G_3 \) is formed. This level stays almost constant even after 19.5 hr of incubation (Fig. 9(c)). On a mole basis, \( G_3 \) accounts for about 3.5% of the cellotriose that was hydrolyzed.

These data indicate that at high enough substrate concentrations, the formation of an \( E^*G \) (or \( EG(GG) \)) type complex will occur and some reversion product, i.e., cellotriose, will form. This result is not surprising since it is well known that at high enough substrate concentrations most enzymes will exhibit substrate inhibition. On

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**Fig. 8.** LPLC chromatogram of cellotriose hydrolysate.
a practical note, however, the cellobiose concentrations commonly encountered from the hydrolysis of cellulose by the cellulase complex from *T. reesei* in our laboratory have typically been 10 mM or less. This range corresponds to the concentration used in generating the hydrolysate for the LPLC chromatogram in Figure 8.

These results show it is possible to predict the time course of cellobiose hydrolysis by glucanohydrolase using equations derived by making the pseudo-steady-state assumption. Again, this approach is different from ordinary curve fitting since the model as well as kinetic parameters were derived from initial rate data. This information was then taken to predict the time course for up to 90% conversion of cellobiose. This is the first time, to our knowledge, that theory and experiment have been combined in this particular
manner to quantitate glucanohydrolase activity with respect to cellulobiose.

**DISCUSSION**

The initial rate pattern for hydrolysis of cellulobiose by glucanohydrolase is different from that for cellulase. Glucanohydrolase exhibits essentially a competitive mechanism, while cellulase exhibits a noncompetitive mechanism. Perhaps more significantly, a comparison of the kinetic parameters for glucanohydrolase and cellulase (see Table II) shows that glucanohydrolase has several magnitudes lower activity with respect to cellulobiose than does cellulase (compare $V/E_{in}$). The comparison is only semiquantitative since the constants were determined at slightly different temperatures, but the difference is still significant. In another paper, a cellulobiodydrolase, the third major component of the cellulose system, was shown to have no activity with respect to cellulobiose. These data, taken together, indicate that the major cellulose-hydrolyzing enzyme in the cellulase system is cellulase, although glucanohydrolase also has some cellulobiose-hydrolyzing activity.

The Michaelis constant, $K_m$, and the competitive inhibition constant, $K_{i}$, are both similar for the two enzymes. Since the kinetic data for the two enzymes were taken at slightly different temperatures (40°C for glucanohydrolase, and 45°C for cellulase), the comparison is again only semiquantitative.

Several methods were used to verify the homogeneity of glucanohydrolase used for the kinetics reported in this paper. The first,

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Glucanohydrolase 1</th>
<th>Cellolase&lt;sup&gt;a&lt;/sup&gt; peak 2</th>
<th>Cellolase&lt;sup&gt;a&lt;/sup&gt; peak 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V/E_{in}$ (mmol glucose/mg protein)</td>
<td>0.58</td>
<td>116</td>
<td>44.6</td>
</tr>
<tr>
<td>$K_m$ (mM cellulose)</td>
<td>1.6</td>
<td>2.5</td>
<td>2.74</td>
</tr>
<tr>
<td>$K_i$ (mM glucose)</td>
<td>not applicable</td>
<td>10.4</td>
<td>14.7</td>
</tr>
<tr>
<td>$K_{i}$ (mM glucose)</td>
<td>0.98</td>
<td>1.22</td>
<td>4.36</td>
</tr>
</tbody>
</table>

<sup>a</sup> Kinetic constants at 40°C.

<sup>b</sup> Data taken from Ref. 10; kinetic constants at 45°C.
SDS-polyacrylamide gel electrophoresis, verified that the enzyme had a uniform molecular weight with no other detectable protein bands present (see Fig. 2). A second indication of the protein homogeneity was obtained from fractionation of the enzyme on Sephadex G-100. The chromatogram (see Fig. 3) shows that the glucanohydrolase peak is symmetrical. The elution volume of this peak was the same as that of a protein marker having a molecular weight of 52,000 daltons.

The antisera against purified glucanohydrolase test was used to test the cross-contamination by celllobiase and celllobiohydrolase by immunodiffusion assay. The results showed no cross-reactions between glucanohydrolase and celllobiase within the resolution of this technique. This indicates that the purified glucanohydrolase was free from celllobiase contamination. Moreover, there was no cross-reaction between glucanohydrolase and celllobiohydrolase. This indicates that the glucanohydrolase was free from celllobiohydrolase contamination as well.

The data on enzyme purity and enzyme kinetics, taken together with previously reported data for celllobiase,\textsuperscript{10} are significant because they show that glucanohydrolase and celllobiase are two different enzymes although both enzymes have celllobiose-hydrolyzing activity. The homogeneity of glucanohydrolase is verified by the SDS-gel, Sephadex G-100, and antibody tests.

The detection of celllobiase contamination of endoglucanase based on the mode of action of the enzyme preparation with respect to cellulose as a substrate is difficult. Other approaches suggested by the literature include inhibition characteristics with respect to nojirimycin,\textsuperscript{11} and gluconolactone\textsuperscript{12} and substrate specificity.\textsuperscript{12}

Nojirimycin is known to be a strong inhibitor of celllobiase. However, no data have been reported for the effect of nojirimycin on glucanohydrolase, to the best of the authors' knowledge. Hence, detection of celllobiase in glucanohydrolase by measuring inhibition by nojirimycin is, by itself, insufficient since both enzymes have activity with respect to celllobiase.

Gluconolactone is also a strong inhibitor of celllobiase. However, gluconolactone is also a strong inhibitor of cellulase enzymes having activity with respect to carboxymethylcellulose (i.e., glucanohydrolase).\textsuperscript{13} Hence, quantitative knowledge of the kinetics of gluconolactone inhibition with respect to both celllobiase and glucanohydrolase is necessary to detect celllobiase in the presence of glucanohydrolase.
Substrate specificity is perhaps the most obvious of the two approaches. Pure cellobiase would be expected to have high activity toward cellobiose while glucanohydrolase would not. However, since both enzymes have cellobiase-hydrolyzing activity, substrate specificity with respect to cellobiose is not sufficient to discern whether small amounts of cellobiase might be present in an otherwise pure glucanohydrolase enzyme fraction. In this case, another criterion, such as kinetic patterns obtained from initial rate data, is needed.

The data presented in this paper and others clearly show that the initial rate kinetic patterns with respect to cellobiose for the two enzymes are different. These patterns represent "finger-prints" for the two enzymes that are helpful, together with other supporting data, to establishing the homogeneity of a glucanohydrolase preparation.

The reaction sequence with respect to cellobiose for the two enzymes together can be represented by

\[
E_1 + GG \xrightarrow{k_{11}} E_1^* \xrightarrow{k_{21}} E_1^* + 2G \\
E_1^* + G \xrightarrow{k_{41}} E_1G \\
E_1 + G \xrightarrow{k_{41}} E_1G \\
E_1 + GG \xrightarrow{k_{31}} E_1^* \xrightarrow{k_{23}} E_1^* + 2G \\
E_1 + G \xrightarrow{k_{41}} E_1G
\]

(10)

where \(E_1\) and \(E_1^*\) represent free cellobiase and glucanohydrolase, respectively; \(E_1^*\) and \(E_1^*\) represent the enzyme-substrate complexes for the two enzymes; and the other symbols represent the quantities as defined previously in this paper for the two respective enzymes.

The rate of formation of glucose is given by

\[
v = \frac{dG}{dt} = 2k_{31}E_1^* + 2k_{31}E_2^* = \frac{dG_1}{dt} + \frac{dG_2}{dt}
\]

(11)

where \(dG_1/dt\) is the rate of glucose formed due to cellobiase and \(dG_2/dt\) is the rate of glucose formed by glucanohydrolase. Substi-
tution of expressions for $dG_1/dt$ (see Ref. 10) and $dG_2/dt$ (derived previously in this paper) gives:

$$v_t = \frac{V_1(GG)}{(1 + G/K_{1,11})(GG) + (1 + G/K_{1,12})K_1}$$

$$+ \frac{V_2(GG)}{K_2(1 + G/K_{2,12}) + GG}$$

(12)

Inverting eq. (12) and rearrangement results in:

$$1/v_t = \frac{1}{K_2(1 + G/K_{2,12}) + GG}$$

$$\left[ \frac{1}{V_1((1 + G/K_{1,11})(1 + G/K_{1,12}) + K_1(1 + G/K_{1,12}))} + \frac{1}{V_2((1 + G/K_{1,11}))(1 + G/K_{1,12} + G)} \right]$$

$$+ \left[ \frac{1}{K_2(1 + G/K_{2,12}) + GG} \right]$$

(13)

Equation (13) is useful in examining the effect of enzyme mixture on the $y$ intercept (i.e., $1/V$) on a Lineweaver–Burk plot. The $y$ intercept is determined by extrapolating to the condition $1/GG \rightarrow 0$ (i.e., $GG$ becomes very large). As $GG$ becomes very large, eq. (13) reduces to:

$$\frac{1}{v_t} = \frac{1}{V} = \frac{1 + G/K_{1,11}}{V_1(1 + G/K_{1,11}) + V_2} = b$$

(14)

where $V$ is the maximum reaction velocity, i.e. the $y$ intercept, $b$, on a Lineweaver–Burk plot.

It is useful to consider several limiting cases of eq. (14).

**Case (i):** $V_1 = 0$ (i.e., enzyme consists of pure glucanohydrolase):

$$b = \frac{1 + G/K_{1,11}}{V_2(1 + G/K_{1,11})} = \frac{1}{V_2}$$

(15)

For this case, the $y$ intercept on the Lineweaver–Burk plot is not a function of glucose concentration $G$ and is therefore constant.

**Case (ii):** $V_2 = 0$ (i.e., enzyme is pure cellobiase):

$$b = \frac{1 + G/K_{1,11}}{V_1}$$

(16)

This predicts that the $y$ intercept will increase with increasing initial
glucose concentration. This is observed for cellobiase (see Refs. 9 and 10).

Case (iii): $V_1 \neq 0$ and $V_2 \neq 0$ (i.e., enzyme is a mixture of cellobiase and glucanohydrolase). In this case the $y$ intercept is given by eq. (14). The $y$ intercept will be a function of initial glucose concentration as well as the relative amounts of the two enzymes.

Equation (14) predicts the type of patterns that arise when small amounts of cellobiase are present in a glucanohydrolase enzyme fraction. Using the data from Table II ($V_1/E_{tot} = 116$, $V_2/E_{tot} = 0.6$, $K_{c.m} = 16.4$), eq. (17) is obtained:

$$b' = \frac{1 + G/16.4}{(1 + G/16.4) + 2x} \cdot \frac{1}{V_2/E_{tot}}$$

(17)

where $x$ is the percent (by protein weight) of cellobiase contamination. Equation (17) predicts that the measured $y$ intercept, $b'$, will increase with increasing glucose concentration. Using values of $G$ of 0, 1.59, and 3.17mM glucose at 1, 2, 5, and 10% cellobiase (i.e., $x = 1, 2, 5,$ and 10, respectively), the pattern of intercepts shown

Fig. 10. Effect of cellobiase in the presence of glucanohydrolase on $y$ intercepts of Lineweaver-Burk plot. Lines for homogeneous glucanohydrolase intersect on the $y$ axis.
in Figure 10 results. Hence, contamination of glucanohydrolase with small quantities of cellubiose will be indicated by an initial rate pattern that does not intersect on the y axis. The pattern for pure glucanohydrolase intersects the y axis at 0.6. A more distinguishing feature is that the "apparent" activity of the glucanohydrolase preparation will decrease with decreasing cellubiose. This is caused by the much higher specific activity of cellubiose with respect to cellubiose relative to glucanohydrolase.

CONCLUSION

Glucan glucanohydrolase from T. reesei has cellubiose-hydrolyzing activity. However, this activity is one to two magnitudes of order less than that observed for cellubiose. Thus, it appears that the enzyme component in the cellulase complex of T. reesei having the major cellubiose-hydrolyzing activity is cellubiose. Although both glucanohydrolase and cellubiose are both subject to glucose (product) inhibition, the mode of inhibition for glucanohydrolase is competitive, while the mode for cellubiose is noncompetitive.

APPENDIX

The reactions for competitive inhibition are:

\[ E + GG \xrightleftharpoons[\kappa_2]{\kappa_1} E^* + G \]
\[ E + G \xrightarrow{\kappa_3} EG \]

where \( E \) represents the free enzyme, \( GG \) represents cellubiose (a substrate for glucanohydrolase), and \( E^* \) and \( EG \) represent enzyme-substrate and enzyme-product (inhibitor) complexes, respectively. Writing out differential equations for \( E^* \) and \( EG \) and applying the pseudo-steady-state assumption results in the equations:

\[ -\frac{d(E^*)}{dt} = 0 = (k_1 + k_3)(E^*) - k_2(E)(GG) \]  (18)
\[ -\frac{d(EG)}{dt} = 0 = k_2(EG) - k_1(E)(G) \]  (19)

Equations (18) and (19), combined together with eq. (20), an equation representing conservation of enzyme:

\[ E_{tot} = E + E^* + EG \]  (20)

results in an expression for \( E^* \):

\[ E^* = \frac{E_{tot}}{1 + \left(\frac{k_3}{k_1}\right)\sqrt{GG}} + \left(\frac{k_2}{k_3}\right)\left(\frac{k_1}{k_2 + k_3}\right)\sqrt{GG} \]  (21)
The rate of appearance of the product glucose is given by:

$$\frac{dG}{dt} = 2k_v E^*$$  \hspace{1cm} (22)

Substitution of eq. (21) into eq. (22) gives:

$$\frac{dG}{dt} = \frac{2k_v E_m}{1 + [(k_2 + k_{1a})/k_c][1/GG] + [(k_1/k_c)][(k_2 + k_{1a})/k_c][G/GG]}$$  \hspace{1cm} (23)

where the constants may be redefined as:

$$K = (k_2 + k_{1a})/k_c, \quad K_{cat} = k_v/k_c, \quad V = 2k_v E_m.$$

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References


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