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A Kinetic Model of the Synergism of Endo- and Exoglucanase and β-Glucosidase on Hydrolysis of Cellulose

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ABSTRACT

A kinetic model representing the synergistic action of the three components that compose cellulase on hydrolysis of solid cellulose particles is proposed.

The model consists of three simultaneous differential equations: one representing the action of the endoenzyme, another representing the action of the exoenzyme, and the third representing the action of the β-glucosidase. A simultaneous solution of these three equations expresses the synergism.

The experimental data fit the theory well.

Index Entries: Enzymatic hydrolysis of cellulose; synergism of cellulase components; endo-β-1,4-glucanase; exo-β-1,4-glucanase; β-glucosidase.

NOMENCLATURE

[C]	Concentration of cellobiose	mol/mL
[E_{en}]	Concentration of EG	U/mL
[E_{enf}]	Concentration of free EG	U/mL
[G]	Concentration of glucose	mol/mL
[C.E_{en}]	Concentration of EG-cellobiose complex	mol/mL
k_{en}	Reaction rate constant of EG	mg/min U

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K_{i1}	Inhibition constant of cellobiose, Eqs. 1 and 3	mol/mL
K_{i3}	Inhibition constant of glucose, Eq. 5	mol/mL
K_{ib}	Inhibition constant of glucose, Eq. 6	mol/mL
K_{i2}	Inhibition constant of cellobiose for EG	mol/mL
K_{i4}	Inhibition constant of glucose for EG	mol/mL
K_m	M-M constant of CBH	mg/mL
K_{mb}	M-M constant of BG	mol/mL
[S]	Concentration of the substrate	mg/mL
[S_0]	Initial concentration of the substrate	mg/mL
t	Reaction time	min
V_m	Maximum velocity of CBH	mol/mL min
V_{mb}	Maximum velocity of BG	mol/mL min

INTRODUCTION

Cellulase secreted by microorganisms consists of endo-β-1,4-glucanase (EG), exo-β-1,4-glucanase (cellobiohydrolase, CBH), and β-glucosidase (BG). During solubilization of solid cellulose particles by celluase, synergism of these three components plays a significant role (*1,2*).

Recently, EG, CBH, and BG have been further divided by Wood (*3*) to get purely isolated enzymes. Interestingly, some combinations of these finely isolated components show synergism and some do not (*4,5*).

From our point of view, discussion of synergism needs a kinetic analysis. Even for a combination of the same species of enzymes, the degree of synergism depends largely on the proportions of the enzymes and on the reaction time (*6*). The above papers are somewhat lacking in kinetic information.

In this article, we construct a model representing the synergism of EG, CBH, and BG, assuming each enzyme's role, and examine the ability of the model to fit the experimental data.

KINETIC MODEL

Assumptions on Which the Kinetic Model Is Constructed

A kinetic model representing the synergistic action of EG, CBH, and BG on solubilizing cellulose particles is constructed on the basis of the following assumptions:

1. Both glucanases, EG and CBH, act only on the surface of the solid substrates.
2. CBH acts only on a nonreducing end of a substrate particle exposed on the surface to form cellobiose. The formation of cellobiose obeys the Michaelis-Menten (M-M) mechanism.

3. In the case in which only CBH is present, the concentration of the substrate for this enzyme can be assumed not to change during the reaction, because, when one cellobiose molecule is removed by CBH, one new nonreducing end group is formed at the same time. However, the reaction does not last long, rather, it seems to stop, since, after the nonreducing end groups on the surface are all exhausted, there exist many fewer end groups exposed on the second layer of the substrate particle.
4. The action of EG apparently does not obey the M-M mechanism, since, when the progress of the reaction is measured in terms of formation of soluble sugar, most of the reaction products that have newly formed nonreducing end groups remain on the surface of the particle. A small amount of soluble sugar could be released from the surface of the particle, but it may be ignored.
5. In a mixed-enzyme system of EG and CBH, partial synergism can be observed. The action of CBH in Assumption 2 is said to be peeling the molecule on the first layer of the particle. As the action proceeds, the new glucoside bonds on the next layer, which can be subject to EG's attack, are exposed. The action of EG on the second layer then supplies newly formed nonreducing end groups for CBH. Beldman et al. (*7*) have suggested this mechanism.
6. The number of the glucoside bonds on molecules on the surface of the substrate particles is so large that we can assume that the rate of the reaction by EG is independent of the substrate concentration as long as the particles are present in the reaction system.
7. In a three-component system, BG acts on cellobiose to form glucose, rendering glucose the sole product, that is, the single inhibitor, in the reaction system. That is another part of the synergism. Because of the presence of BG, cellobiose inhibition for CBH and EG in the mixed two-component system is released and changes to the less effective glucose inhibition. Halliwell et al. (*1*) discussed this possibility.

The Kinetic Equations

The kinetic equations derived from the model are shown as follows:

1. The expression for the sole CBH system, in accordance with Assumptions 2 and 3, is given by

$$d[C] / dt = V_m[S_0] / \{K_m(1+[C]/K_{i1}) + [S_0]\} \quad (1)$$

where $[C]=0$ at $t=0$ and $[S_0]$ is the initial mass concentration of substrate particles, assumed to be proportional to the num-

ber of nonreducing end groups initially exposed on the surface of the particles.

2. For the mixed EG and CBH systems, the simultaneous differential equations are as follows:

$$d[S] / dt = k_{en}[E_{en}] \{1 - [C] / ([C] + K_{i2})\} \text{ (cf Appendix)} \quad (2)$$

$$d[C] / dt = V_m[S] / \{K_m(1 + [C]/K_{i1}) + [S]\} \quad (3)$$

where $[S]=[S_0]$ and $[C]=0$ at $t=0$, and [S] designates the concentration of the substrate, which is essentially a sum of the nonreducing end groups of the original on the surface and of those newly formed by the action of EG.

3. For the mixed EG, CBH and BG systems, glucose is the sole product, and the following three simultaneous differential equations hold:

$$[G] / ([G] + K_{i4}) \text{ is substituted for } [C] / ([C] + K_{i2}) \text{ in Eq. 2.} \quad (4)$$

$$[G] / K_{i3} \text{ is substituted for } [C] / K_{i1} \text{ in Eq. 3.} \quad (5)$$

$$d[G] / dt = V_{mb}[C] / \{K_{mb}(1 + [G]/K_{ib}) + [C]\} \quad (6)$$

where the initial conditions are the same as those for Eqs. 2 and 3, except $[G]=0$ at $t=0$.

EXPERIMENTAL

Materials

Substrate

Avicel (Avicel SF for thin-layer chromatography, lot no. 8387, Asahi Kasei Kogyo K.K., Tokyo, Japan) was used as the substrate.

Enzymes

Meicelase (a commercial product of cellulase from *Trichoderma koningii*, a gift from Meijiseika K.K., Tokyo, Japan) was fractionated by the procedure described below. Novozyme 188 (a commercial product of BG from *Aspergillus niger*, a gift from Novo Industries Japan Ltd., Tokyo, Japan, lot no. DCN 0003) was fractionated by the same procedure as used for the fractionation of Meicelase.

Procedures

Fractionation of the Enzymes

An enzyme was dissolved in a 0.01*M* acetate buffer (pH 5.0), and the enzyme solution was filtered through an ultrafiltration membrane (Toyo Roshi K.K., Tokyo, Japan UK-10) to desalt and concentrate the enzyme

solution. The concentrated enzyme solution was then subjected to fractionation by ion-exchange chromatography using a DEAE-Sephadex A-50 column (diameter 2.5 cm, length 80 cm) that was previously equilibrated with 0.01*M* acetate buffer. The elution was done with the same buffer, with an increased linear gradient of ionic strength in the range 0–0.5*M* with respect to sodium chloride. Both enzymes were fractionated by the same procedure, except that the ultrafiltration step for Novozyme was omitted because of plugging on the filter membrane.

Enzyme Assays

One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of reducing sugar for both EG and CBH, or 1 μmol of *p*-nitrophenol for BG, per minute.

CARBOXYMETHYLCELLULASE (CMCASE) ASSAY

A sample solution (0.5 mL) was added to 1 mL of a CMC solution (10 mg of CMC dissolved in 1 mL of 0.1*M* acetate buffer, pH 5.0), and the mixture was kept at 30 ± 1°C for 20 min. Then 1 mL of the reaction mixture was added to 2 mL of the Somogyi copper reagent (*8*) and boiled for 10 min to stop the reaction. After cooling, 2 mL of Nelson reagent (*8*) was added, with agitation. After 5 mL of water was added, the whole mixture was centrifuged to remove flocky substrate materials deposited. The absorbance at 660 nm was measured for the supernatant.

AVICELASE ASSAY

A sample solution (0.5 mL) was added to 3 mL of an Avicel suspension (50 g of Avicel was equilibrated in 1 L of a 0.1*M* acetate buffer (pH 5.0) for 24 h) in an L-shaped test tube. The test tube was incubated, with vibration, at 50 ± 1°C for 60 min. After the reaction was stopped by immersing the test tube in a boiling water bath for 5 min, the substrate was removed by centrifuging. The reducing sugar in the supernatant liquid was measured by the same procedure as used for CMC activity measurement, above.

BG ASSAY

Activity toward *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG) (Nakarai Chemicals Ltd., Kyoto, Japan) was determined in terms of *p*-nitrophenol released from *p*-NPG in a reaction mixture of 0.1 mL of a sample solution, 0.5 mL of 5 m*M* *p*-NPG, and 1 mL of 0.1*M* acetate buffer (pH 5.0). After incubation at 30°C for 20 min, 1.0 mL of 1.0*M* sodium carbonate was added to the mixture and the amount of *p*-nitrophenol liberated was estimated from the absorbance at 420 nm. (*9*).

Determination of Protein

Protein in the fractionated solution of column chromatography was determined by the Lowry-Folin method (*10*) with crystalline bovine serum albumin as a standard.

Determination of Soluble Sugar

REDUCING SUGAR

Total reducing sugar was determined by the Smogyi-Nelson method (*8*).

GLUCOSE

The glucose-C test (Wako Junyaku Kogyo K.K., Osaka, Japan) (*11,12*) was used.

Concentration of the Enzymes Used in the Hydrolysis Experiments

The concentrations of CBH, EG, and BG used in the hydrolysis experiments were chosen on the basis of the number of activity units of Avicelase, CMCase, and BG activities per volume, respectively.

Procedure for the Hydrolysis Experiments

A prescribed amount of the substrate was suspended in 12.3 mL of 0.1*M* acetate buffer (pH 5.0), and the suspension was agitated for over 24 h in order to equilibrate the cellulose sample in an L-shaped test tube. Then 2.7 mL of an enzyme solution was added, and the reaction mixture was reciprocally shaken at 30±1°C for a prescribed reaction time. After the reaction, 1 mL of the reaction mixture was extracted, and the enzyme was deactivated in a boiling water bath for 10 min and centrifuged to remove the substrate (3000 rpm, 5 min). Reducing sugar and glucose in the supernatant liquid were determined.

RESULTS AND DISCUSSION

Fractionation of Meicelase

Figure 1 shows the anion-exchange chromatogram of Meicelase: Peak 1 was a mixture of BG and CMCase, Peak II had a high CMCase activity, and Peak III had a high Avicelase activity. SDS-gel electrophoresis of Peak II and Peak III revealed that, in each peak, a major component was condensed as a dense band. Since they seemed to be practically pure enough for the present study, we used the fraction of Peak II as EG and the fraction of Peak III as CBH in the hydrolysis experiment. The ratio of Avicelase activity to CBCase activity of each peak is listed in Table 1.

Since we failed to purify BG from Peak 1, in which high BG activity and high CBCase activity were closely overlapped, BG preparation for use was obtained by fractionation of Novozyme. The fractionation procedure was the same as that used for fractionating Meicelase. The BG activity of the purified sample was 16-fold, as high as that of the original Novozyme.

Evaluation of the Reaction Constants

The Lineweaver-Burk plots (*13*) of reducing sugar produced by CBH in 30 min of reaction time for Avicel showed a good linearity, indicating

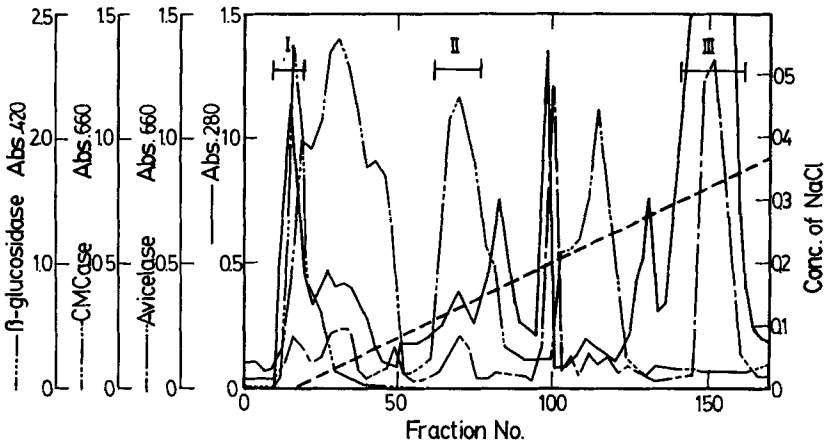

Fig. 1. Fractionation profiles of Meicelase: (----)concentration gradient of NaCl(*M*): I, II, and III designate Peak I, Peak II, and Peak III in Table 1, respectively.

Table 1
CMCase, Avicelase and β-glucosidase Activities of Each Fraction From Meicelase and Novozyme

	CMCase activity U/mg protein	Avicelase activity	β -glucosidase activity
Crude Meicelase	0.13	0.05	0.92
Peak I	1.19	0.24	30.38
Peak II	3.70	0.54	0.39
Peak III	0.01	0.13	0.04
Crude Novozyme	0.14	0.06	0.93
Fractionated Novozyme	0.79	0.46	16.21

that the action of CBH obeys the M-M mechanism. The values of the maximum velocity, V_m, and the M-M constant, k_m, are listed in Table 2.

The unit of the original concentration of the substrate is taken as mg/mL. The number of the nonreducing end groups exposed on the surface of the substrates, which are the objects for the exoenzyme attack, is assumed to be proportional to the mass of the substrate. Therefore, the reaction constants in the M-M equation, V_m and K_m in Eqs. 1, 3, and 5, may be different among different kinds of substrates, depending on their origins or manufacturing processes.

In the mixed EG-CBH systems, cellobiose is the product, and it inhibits the actions of EG and CBH. The inhibition constant of cellobiose for CBH, K_{i1}, was estimated from the Dixon plot (*14*) for the reaction of CBH, Avicel as the substrate, and cellobiose as the inhibitor for 30 min of reaction time. Estimation of the inhibition constant for EG, K_{i2}, is discussed later.

In the mixed three-component systems, glucose produced by BG inhibits the actions of EG, CBH, and BG. The inhibition constant for CBH,

Table 2
Kinetic Constants and Parameters Measured and Used in Calculations

EG		
k_{en} [mg/min.U]	K_{i2} [mol/mL]	K_{i4} [mol/mL]
995	2.65×10^{-9}	7.22×10^{-8}

CBH			
K_m [mg/mL]	V_m [mol/mL.min]	K_{i1} [mol/mL]	K_{i3} [mol/mL]
45.15	9.91×10^{-8}	8.00×10^{-8}	5.60×10^{-8}

BG		
K_{mb} [mol/mL]	V_{mb} [mol/mL.min]	K_{ib} [mol/mL.min]
6.65×10^{-7}	2.47×10^{-7}	2.69×10^{-7}

K_{i3}, was estimated in the same way as K_{i1} with glucose as the inhibitor. The inhibition constant for EG, K_{i4}, was estimated in the same way as K_{i2}.

The Lineweaver-Burk plots for EG did not show a straight line, indicating that formation of soluble reducing sugar by the action of EG does not obey the M-M mechanism. This fact suggests that the main role of EG is hindered in the substrate, that is, the reaction products remain in the substrate.

EG attacks β-glucoside bonds at random in the surface of the substrate particles (Assumption 4). As long as the solid particles are present in the reaction medium, the number of bonds must be large enough to give the reaction first-order kinetics with respect to the enzyme concentration. Thus, the reaction constant k_{en} was determined in the following way:

In simultaneous differential Eqs. 2 and 3, at an early stage of the reaction, the term in brackets in Eq. 2 can be neglected. On substitution of [S] from Eq. 2 into Eq. 3, the first-order differential equation is obtained. Substituting the experimental data for 30 min of a reaction into the solution of the differential equation, we can get a k_{en} value. The value in Table 2 is an average of values for several runs.

As stated previously, [S] in Eqs. 2–5 designates the concentration of nonreducing end groups of cellulose molecules located on the surface of the particles. We have no way to measure these groups, because they remain on the surface of the substrate (Assumption 4). Therefore, K_{i2} and K_{i4} were determined in such a way that simulation of various [C] values for Eq. 3 and of various [G] values for Eq. 6 were applied to the simultaneous solutions of Eqs. 2 and 3 or of Eqs. 4 and 5, respectively, to find the values of K_{i2} or K_{i4} that best fit the experimental data, using the Runge-Kutta method. These values are also listed in Table 2. We find that K_{i4} is four times as large as K_{i2} and that K_{i3} is 1.5 times as large as K_{i1}. This means that inhibition of cellobiose to EG is appreciably released by the action of BG; in other words, the solubilization of solid cellulose is enhanced by the presence of BG (Assumption 7).

Values of k_{mb} and V_{mb} were determined by an experiment making the Lineweaver-Burk plot, in which cellobiose was treated by BG for 10 min, and K_{ib} was estimated from the Dixon plot, in which glucose is the inhibitor in the reaction system of cellobiose and BG. These values are listed in Table 2.

Comparison of the Theoretical Curves with the Experimental Data

Figure 2 compares the amount of soluble reducing sugar produced by enzymatic hydrolysis in the mixed EG–CBH system with that produced in each EG and CBH system. The dotted and solid lines are the curves theoretically calculated by Eq. 1 and by simultaneous Eq. 2 and 3, respectively. The experimental data fit the theoretical curves well, and the synergism is shown.

As we have discussed elsewhere (*6*), the degree of synergism in the mixed EG–CBH system depends on the concentration (U/mL) ratios of both enzymes. That is, there exists an optimum ratio, and, if the mixing ratio is inadequate (one component is in excess), synergism is not present. A simulation of a simultaneous solution of Eqs. 2 and 3 can demonstrate the above facts (*6*).

Figure 3 exhibits a comparison between the amount of glucose produced in a mixed three-component system of EG, CBH, and BG and the amount of reducing sugar solubilized by a mixed EG–CBH system that is the same as shown in Fig. 2. We confirmed that the soluble reducing sugar in the reaction media for the three-component systems was almost glucose. Synergism in the presence of BG appears five times as large as that without BG. The effect of the shift of K_{i2} to K_{i4} is significant, meaning that BG enhances the rate of solubilization of solid cellulose by EG and CBH by releasing the cellobiose's inhibition of both enzymes and changing the inhibitor to the less-active glucose.

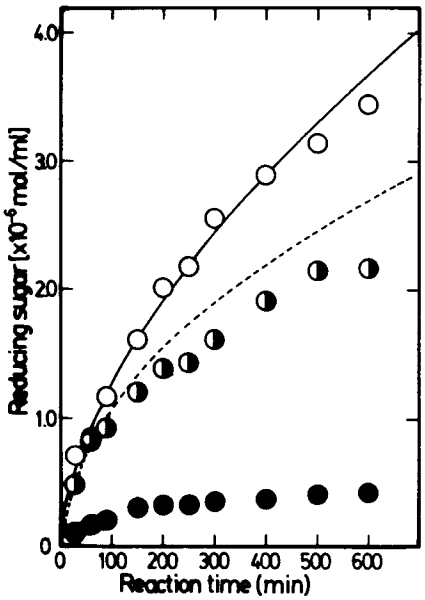

Fig. 2. Comparison of time-course hydrolysis experiments by a sole CBH (1.38×10^{-2} U/mL) system, a sole EG (1.27×10^{-2} U/mL) system, and a mixed system of both enzymes at the same concentrations as in the sole systems. The initial concentration of the substrate Avicel is always 41 mg/mL; (●); (◑), and (○) designate the experimental values for EG, CBH, and their mixture, respectively; the dotted line is the curve calculated from Eq. 1; the solid line is the curve drawn from the simultaneous solution of Eqs. 2 and 3.

CONCLUSIONS

A kinetic model representing the synergism of the three components, EG, CBH, and BG, that compose cellulase was proposed. The model consists of three simultaneous differential equations; each equation expresses each enzyme's action.

Experimental data fitted the theoretically calculated curves well, that is, the synergism is cooperation of the three enzymes, which is executed by the action of each enzyme.

APPENDIX

Cellobiose and glucose are the sole inhibitors in the mixed EG–CBH systems and in the mixed EG–CBH–BG systems, respectively. Each of them inhibits the reaction of every enzyme participating in the reaction system.

Let us take cellobiose as the sole inhibitor in the two-component system, for example.

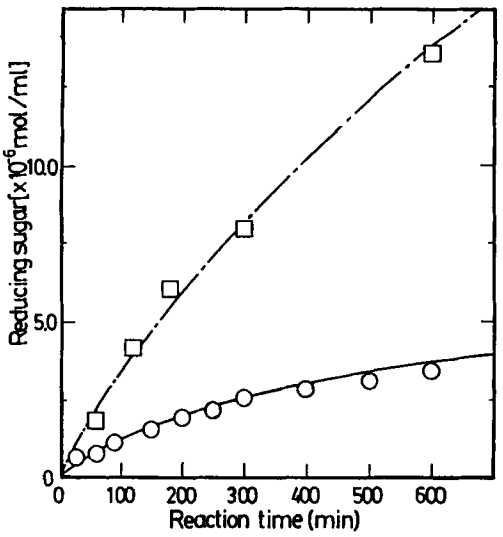

Fig. 3. Comparison of the mixed EG–CBH system (shown by the same symbol and line as in Fig. 2) with a mixed EG–CBH–BG system in which the concentrations of EG and CBH are the same as in the two-component system shown in Fig. 2, and the concentration of BG is 0.175 U/mL. The concentration of Avicel is the same as Fig. 2; (□) designates the experimental data for the three-component system, and the broken line is the curve drawn from the simultaneous solution of Eqs. 4, 5, and 6.

The reaction mechanism for EG can be assumed as follows:

$$[S_0] + [E_{enf}] \underset{}{\overset{k_1}{\rightleftharpoons}} [S] + [E_{enf}] \tag{7}$$

and

$$[C] + [E_{enf}] \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} [C.E_{.en}] \tag{8}$$

where

$$[E_{enf}] = [E_{en}] - [C.E_{en}] \tag{9}$$

The rate of increase is [S] is deduced from Eq. 7 as

$$d[S] / dt = k_{en}[E_{enf}] = k_{en}([E_{en}] - [C.E_{en}]) \tag{10}$$

where $k_{en} = k_1[S_0]$.

The rate of formation of $[C.E_{en}]$ is expressed by

$$\begin{aligned} d[C.E_{en}] / dt &= k_2[C][E_{enf}] - k_{-2}[C.E_{en}] \\ &= k_2[C][E_{en}] - k_2[C][C.E_{en}] \\ &\quad - k_{-2}[C.E_{en}] \end{aligned} \tag{11}$$

Assuming a pseudo-steady-state condition, we get

$$[C.E_{en}] = k_2[E_{en}][C] / (k_2[C] + k_{-2}) \quad (12)$$

On substituting Eq. 12 into Eq. 10, Eq. 10 becomes

$$d[S] / dt = k_{en}[E_{en}] \{1 - [C] / ([C] + K_{iz})\} \quad (13)$$

which is Eq. 2, where $K_{i2} = k_{-2}/k_2$.

The inhibition constant of glucose for the reaction of EG in the three-component systems, K_{i4}, can be deduced in the same manner.

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