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Discrimination Among Eight Modified Michaelis-Menten Kinetics Models of Cellulose Hydrolysis With a Large Range of Substrate/Enzyme Ratios

Inhibition by Cellobiose

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Abstract

The kinetics of exoglucanase (Cel7A) from *Trichoderma reesei* was investigated in the presence of cellobiose and 24 different enzyme/Avicel ratios for 47 h, in order to establish which of the eight available kinetic models best explained the factors involved. The heterogeneous catalysis was studied and the kinetic parameters were estimated employing integrated forms of Michaelis-Menten equations through the use of nonlinear least squares. It was found that cellulose hydrolysis follows a model that takes into account competitive inhibition by cellobiose (final product) with the following parameters: K_m = 3.8 m*M*, K_{ic} = 0.041 m*M*, k_{cat} = 2 h^{-1} (5.6×10^{-4} s^{-1}). Other models, such as mixed type inhibition and those incorporating improvements concerning inhibition by substrate and parabolic inhibition, increased the modulation performance very slightly. The results support the hypothesis that nonproductive enzyme substrate complexes, parabolic inhibition, and enzyme inactivation (Selwyn test) are not the principal constraints in enzymatic cellulose hydrolysis. Under our conditions, the increment in hydrolysis was not significant for substrate/enzyme ratios <6.5.

Index Entries: Cellulase kinetics; cellobiose inhibition; exoglucanase Cel7A; integrated Michaelis-Menten equations; *Trichoderma reesei*; enzyme substrate complex.

Introduction

Cellulose is a renewable resource that can provide economic and environmental benefits *(1)*. The kinetics of enzymatic cellulose saccharifica-

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tion is still under discussion and has led researchers to develop different hydrolysis models *(2)*. The main reason for the observed rate-retarding effect during cellulose hydrolysis has been related to enzyme denaturation *(3)*, transformation of substrate into a less digestible form *(4)*, or end product inhibition. The inhibition pattern exhibited by cellulases remains poorly understood. Lee and Fan *(5)* suggested uncompetitive inhibition by cellobiose and glucose. Pereira *(6)* considered parabolic inhibition by cellobiose. Kim et al. *(7)*, Nidetzky et al. *(8)*, and Eriksson et al. *(9)* considered the possibility of some enzyme cellulose complexes being productive and others being nonproductive. However, competitive *(3)* and noncompetitive inhibition *(10)* are the most readily accepted forms.

The aim of the present work was to investigate the kinetics of exoglucanase Cel7A (formerly known as cellobiohydrolase I, CBHI) with the integrated Michaelis-Menten equations. The reason for focusing on the integrated Michaelis-Menten equations is related to the applicability of this methodology to the kinetic study of enzymatic cellulose hydrolysis *(3,10–16)*.

Materials and Methods

Preparation of Enzyme and Chemicals

Celluclast 1,5 L, a commercial cellulase preparation from *Trichoderma reesei*, was kindly provided by Novo Nordisk A/S (Copenhagen, Denmark). Avicel, carboxymethylcellulose sodium salt, cellobiose, and *p*-nitrophenyl-β-D-glucopyranoside were purchased from Merck. Other reagents were of analytical grade.

Purification of Exoglucanase Cel7A

Exoglucanase Cel7A (EC 3.2.1.91) was purified from Celluclast 1,5 L. To obtain the Cel7A fraction free of other components, we followed a procedure adopted by Beldman et al. *(17)* and Pereira *(6)*. Initial gel filtration chromatography with Bio-Gel P10 was accomplished with 50 m*M* phosphate buffer, pH 7.0, with 0.01% (w/v) sodium azide (buffer A). Two fractions (I.1 and I.2) were obtained. The main cellulolytic activity (fraction I.1) was concentrated by ultrafiltration in an Amicon stirred-cell apparatus fitted with a 10,000 mol wt cutoff membrane and then submitted to DEAE Bio-Gel A (Bio-Rad) anion-exchange chromatography. The column was washed with buffer A and then eluted with a phosphate linear gradient from buffer A to buffer B (300 m*M* phosphate buffer, pH 7.0, 0.01% [w/v] sodium azide). Four fractions (I.1.a, I.1.b, I.1.c, and I.1.d) were obtained. The main Avicelase activity fraction (I.1.d) was pooled and reequilibrated in 50 m*M* citrate buffer, pH 4.8, with 0.01% (w/v) sodium azide (buffer C).

Subsequent affinity chromatography was carried out using a column packed with Avicel equilibrated with buffer C. The column was then sequentially eluted with 500 mL of buffer C, distilled water, and 0.02% (w/v) NaOH. The whole process was carried out at 4°C. The amount of protein in

Table 1
[*S*]/[*E*] Ratios[a]

	[*S*] = 0.025%	[*S*] = 0.25%	[*S*] = 2.5%	[*S*] = 5%
[*E*] = 4 μg/mL	[*S*]/[*E*] = 69	[*S*]/[*E*] = 688	[*S*]/[*E*] = 6875	[*S*]/[*E*] = 13,750
[*E*] = 10 μg/mL	[*S*]/[*E*] = 28	[*S*]/[*E*] = 275	[*S*]/[*E*] = 2750	[*S*]/[*E*] = 5500
[*E*] = 42 μg/mL	[*S*]/[*E*] = 7	[*S*]/[*E*] = 66	[*S*]/[*E*] = 655	[*S*]/[*E*] = 1310
[*E*] = 100 μg/mL	[*S*]/[*E*] = 3	[*S*]/[*E*] = 28	[*S*]/[*E*] = 275	[*S*]/[*E*] = 550
[*E*] = 417 μg/mL	[*S*]/[*E*] = 0.7	[*S*]/[*E*] = 7	[*S*]/[*E*] = 66	[*S*]/[*E*] = 132
[*E*] = 1000 μg/mL	[*S*]/[*E*] = 0.3	[*S*]/[*E*] = 3	[*S*]/[*E*] = 28	[*S*]/[*E*] = 55

[a]Substrate concentration (Avicel) is transformed in potential cellobiose (μg/mL). To fit the experimental values with integrated Michaelis-Menten equations, [*S*] (μg/mL)/[*E*] (μg/mL) ratios <6.5 were not considered.

the pooled fractions was determined by the Lowry method *(18)* using crystalline bovine serum albumin as a standard. The enzyme used in the kinetic studies was the fraction I.1.d.β (Cel7A) obtained when eluted with water. Sodium dodecyl sulfate polyacrylamide gel electrophoresis on a gradient 7–11% polyacrylamide gel was applied to check enzyme purity using the Laemmli discontinuous buffer system.

Enzyme Activities

All enzyme activities were assayed in 50 m*M* citrate buffer, pH 4.8. The hydrolytic activity of the enzyme solution on CMCase, Avicelase, and aryl-β-glucosidase was measured by the IUPAC-Biotechnology Commission procedure *(19)*. The total amount of reducing sugars released was determined by dinitrosalicylic acid or the Somogy-Nelson method, using cellobiose as a standard. The release of *p*-nitrophenol was measured by its absorbance in alkaline solution at 430 nm and converted into a concentration through a standard curve.

Kinetic Assays

Kinetic studies were performed at 40°C in 50 m*M* citrate buffer, pH 4.8. The assays were monitored for 47 h and contained four substrate (Avicel) concentrations (5.000, 2.500, 0.250, and 0.025%) equivalent to 154.2, 77.1, 7.7, and 0.77 m*M* potential cellobiose and six Cel7A concentrations (4, 10, 42, 100, 417, and 1000 μg/mL) according to the combinations reported in Table 1. Identical experiments with added initial cellobiose (7.5 and 15.0 m*M*) were conducted at the 5.0 and 2.5% substrate concentrations and with enzyme concentrations of 1000 and 100 μg/mL under the same temperature and pH conditions as just described.

Kinetic constants for the models presented in Fig. 1 were determined by analysis of the entire progress curve at all experimental points (ratios of substrate [*S*] [μg/mL]/enzyme [*E*] [μg/mL] <6.5 were not considered; Table 1) using the nonlinear regression method DUD (does not use derivatives) of SAS software (SAS, Cary, NC).

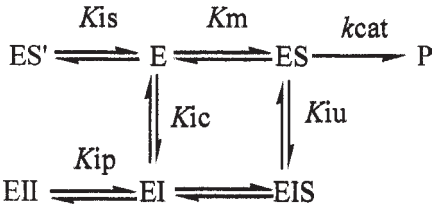

Fig. 1. Mixed linear (total) inhibition model with substrate and parabolic inhibition (MISPI) in which *E* is the enzyme; *ES* is the enzyme substrate complex; *S*,*S* is the substrate (cellulose); *EIS* is the enzyme substrate inhibitor complex; *EI*, *EII* is the enzyme inhibitor complex; *P* is the product (cellobiose); K_m is the Michaelis-Menten constant; K_{ic}, K_{iu}, K_{ip}, and K_{is} are inhibition constants; and k_{cat} is the rate constant. A model without inhibition (WI) can be obtained by simplification of the previous model assuming K_{is}, K_{ip}, K_{ic}, and K_{iu} as infinite. Thus, it is possible to obtain the following linear (total) inhibition models: mixed inhibition model with parabolic inhibition (MIPI) ($K_{is} = \infty$); mixed inhibition model with substrate inhibition (MISI) ($K_{ip} = \infty$); mixed inhibition model (MI) (K_{ip} and $K_{is} = \infty$); competitive inhibition model (CI) (K_{iu}, K_{ip}, and $K_{is} = \infty$); noncompetitive inhibition model (NCI) ($K_{ic} = K_{iu}$, K_{ip} and $K_{is} = \infty$); uncompetitive inhibition model (UCI) (K_{ic}, K_{ip}, and $K_{is} = \infty$).

Results and Discussion

Theoretical Framework

The linear mixed inhibition model with substrate and parabolic inhibition (MISPI) (Fig. 1) is based on the assumption that cellulases may form productive and nonproductive enzyme substrate (*ES*) complexes. While the formation of productive *ES* complexes leads to cellulose hydrolysis and the concomitant release of enzyme and cellobiose, nonproductive *ES* complexes are catalytically inactive. Thus, it is hypothesized that the presence of a productive and nonproductive enzyme-substrate complex reflects two distinct interactions between the enzyme and the substrate but not the transformation of the substrate structure itself. This hypothesis was considered in general terms by Klyosov and Rabinovich *(20)*, Nidetzky et al. *(21)*, and Väljamäe et al. *(22)*.

The reason for considering the parabolic inhibition was owing to the knowledge that catalytic domains of Cel7A, and the exocellulases in general, have particular three-dimensional structures and a long, tunnel-shaped active site with several binding subsites *(23)*. It is not surprising, therefore, that the model assumes a parabolic inhibition once a minimum of two cellobiose molecules bind to the active site.

Assuming the general model (MISPI) in Fig. 1 and the Michaelis-Menten kinetics, we obtain the following rate equation *(24)*:

$$v = \frac{VS_0}{K_m\left(\left(1+\frac{S_0}{K_{is}}\right)+\frac{P}{K_{ic}}\left(1+\frac{P}{K_{ip}}\right)\right)+S_0\left(1+\frac{P}{K_{iu}}\right)} \quad (1)$$

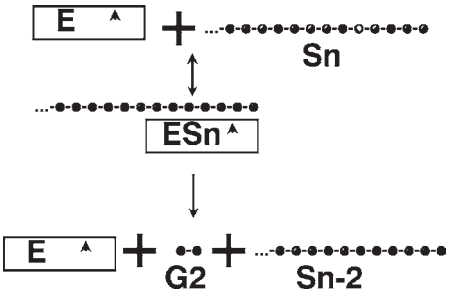

Fig. 2. Diagram of cellulose hydrolysis by CBHs showing constant substrate concentration during reaction. G2 is the cellobiose molecule; Sn is the cellulose molecule with *n* glucopyranosyl monomers; E is the enzyme; Sn-2 is the cellulose molecule without one cellobiose. The symbol ^ shows the active site of the enzyme.

The enzyme Cel7A performs a processive movement along cellulose chains, which may have thousands of cellobiose units *(23)*. Since Cel7A releases reducing sugars with no significant decrease in the number of reaction sites (Fig. 2) *(25)*, the rate equation was integrated assuming the substrate constant

$$V\int_0^t dt = \left(\frac{K_m}{S_0} + \frac{K_m}{K_{is}} + 1\right)\int_{P_0}^{P_t} dP + \left(\frac{K_m}{S_0 K_{ic}} + \frac{1}{K_{iu}}\right)\int_{P_0}^{P_t} P\,dP + \frac{K_m}{S_0 K_{ic} K_{ip}}\int_{P_0}^{P_t} P^2\,dP \tag{2}$$

$$t = \frac{1}{V}\left\{\left(\frac{K_m}{S_0} + \frac{K_m}{K_{is}} + 1\right)(P_t - P_0) + \left(\frac{K_m}{S_0 K_{ic}} + \frac{1}{K_{iu}}\right)\frac{1}{2}(P_t^{\,2} - P_0^{\,2}) + \left(\frac{K_m}{K_{ic} K_{ip} S_0}\right)\frac{1}{3}(P_t^{\,3} - P_0^{\,3})\right\}$$

in which *S* (substrate) is cellulose; *P* (product) is cellobiose; $[P_t]$ is the product concentration at time *t*; and the subscript *o*, such as $[S_o]$ and $[P_0]$ is the initial concentration at $t = 0$.

The seven other kinetic equations are simplifications of this one. For example, if K_{ip} is negligible ($K_{ip} = \infty$), the equation does not contain the last term $(K_m/(K_{ic}K_{ip}S_0)1/3(P_t^{\,3} - P_0^{\,3})$ and the model obtained is MISI (mixed linear inhibition with substrate inhibition). All the models are different forms of the Michaelis-Menten equation assuming different types of inhibition.

Another explanation for the slowdown of cellulose hydrolysis could be that the available substrate concentration effectively decreased in the course of the reaction. Nevertheless, when the velocity equations were integrated assuming the decrease in substrate, similar values for the inhibition kinetic constants were obtained *(14)*.

There are several advantages to using integrated equations, but the possibility of determining kinetic constants when the substrate concentration is not well known is worthy of special mention *(26,27)*. This is

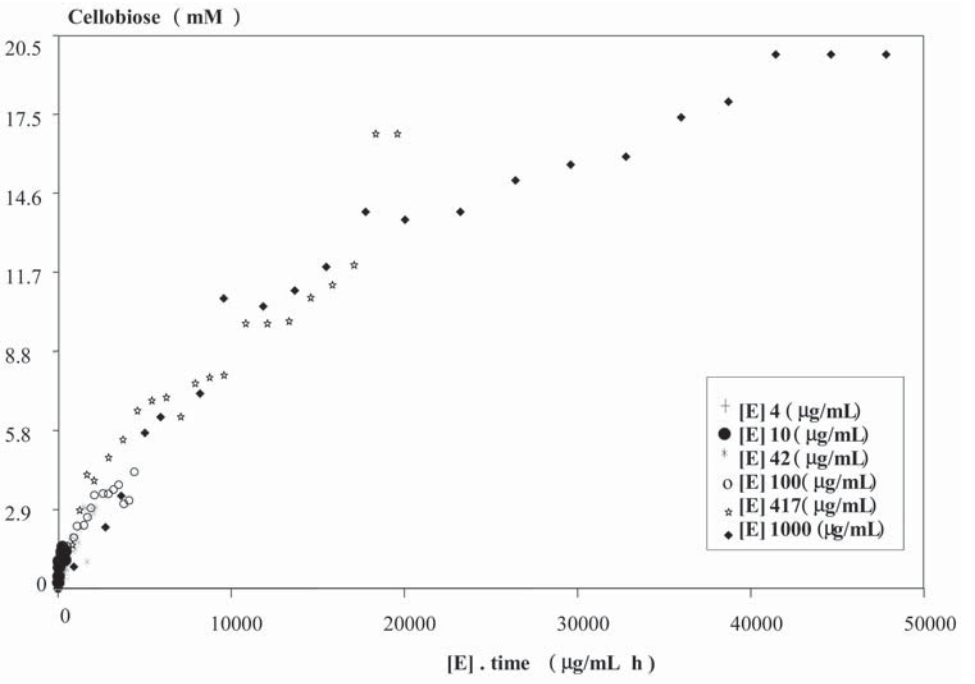

Fig. 3. Representation of Selwyn's graphic. Experimental results were obtained with exoglucanase Cel7A and 2.5% (w/v) Avicel without initial cellobiose. The results show that there was no significant enzyme deactivation.

especially important when the substrate is insoluble cellulose. Other advantages are (1) that more information can be obtained from each experiment, resulting in savings of both time and materials; (2) that the initial addition of end product inhibitor is eliminated, thus preventing possible errors related to incorrect inhibitor stereochemistry or reagent impurities; and (3) that the need to measure rates is eliminated. In the particular case of cellulose hydrolysis, the determination of initial rates can be affected by experimental errors, which are increased in the presence of initial cellobiose added *(28–34)*.

Stability of Cel7A

The integrated equations assume that the only reason for a decrease in velocity is related to substrate depletion or enzyme inhibition, such as end product inhibition. Hsu *(15)* showed that integrated equations could be utilized to study cellulose kinetics if no enzyme denaturation or reaction reversibility occurs during the time course reaction. Nevertheless, reaction reversibility is not a major constraint on the utilization of integrated equations *(35)*. To further investigate enzyme stability under reaction conditions, we applied the Selwyn test *(36)* of enzyme denaturation. Progress curves with six Cel7A concentrations are shown in Fig. 3. Since [*P*] is a function of [*E*] multiplied by time, if there were denaturation then the

Table 2
Summary of the Obtained Constants
and Statistical Parameters in Assays With Cel7A[a]

	WI	CI	NCI	UCI	MI	MIPI	MISI	MISPI
K_m (m*M*)	4.4	3.8	9.3	8.2	3.8	3.8	3.8	3.8
K_{ic} (m*M*)		0.041	1.3		0.044	14.5	14.5	14.5
K_{iu} (m*M*)			1.3	1.4	64.3	64.3	64.3	64.3
K_{ip} (m*M*)						8.8×10^{11}		6×10^9
K_{is} (m*M*)							2.6×10^6	6×10^8
k_{cat} (h^{-1})	1	2	2	2	2	2	2	2
R^2	0.49	0.81	0.73	0.72	0.81	0.81	0.81	0.81
SSE	162,021	59,439	84,892	88,673	59,439	59,431	59,439	59,431
p	2	3	3	3	4	5	5	6
n	563	563	563	563	563	563	563	563

[a]The models are explained in Fig. 1; see also the text.

Selwyn plot would show several curves with different enzyme concentrations. From the results in Fig. 3, it is evident that this did not occur in our study. Under our conditions, the enzyme was fully stable during the time course of cellulose hydrolysis (Fig. 3), which is in agreement with the findings of other investigators *(9)*.

Modeling of Cellulose Hydrolysis

The parameters of the kinetic models were estimated through the use of the DUD algorithm that gives (except for the additional evaluations needed for starting) one iteration for each function evaluation under normal operation. The performance of this algorithm compares favorably with even the best derivative-based algorithms *(37)*. The method has the further advantage of being readily amenable to direct on-line computer analysis of the results by essentially simple procedures.

To fit the experimental values with integrated Michaelis-Menten equations, [*S*] (μg/mL)/[*E*] (μg/mL) ratios <6.5 were not considered (Table 1), since the models do not perform well with these ratios and, under such conditions, the Michaelis-Menten assumption [free substrate] = [total substrate] is not valid *(24)*. The kinetic investigation consisted of two parts: discrimination among available models and parameter estimation (Tables 2 and 3). When two models, e.g., *A* and *B* with p_A and p_B parameters are fitted (separately) to the same data set with *n* experimental points, the model giving the lowest *SSE* (sum of squares error) value should normally be regarded as giving the "best" fit and is a measure of the goodness of fit of the mathematical model to the data set *(38)*. If the models' SSE, SSE_A and SSE_B, differ only by a small amount, the question arises, is the difference large enough for discrimination to be possible? Since, in this case, model *B* would be an extension of the simpler model *A*, the degree of improvement obtained by the addition of the new parameters ($p_B - p_A$) can be tested by

Table 3
Summary of $w - f_{0.95}$ Values in Assays With Cel7A[a]

Models A/B	SSE_A	SSE_B	n	p_A	p_B	w	$p_B - p_A$	$n - p_B$	$f_{0.95}$	$w - f_{0.95}$ [b]
CI/MI	59,439	59,439	563	3	4	0.000	1	559	3.84	–3.840
CI/MIPI	59,439	59,431	563	3	5	0.038	2	558	3.00	–2.962
CI/MISI	59,439	59,439	563	3	5	0.000	2	558	3.00	–3.000
CI/MISPI	59,439	59,431	563	3	6	0.025	3	557	2.60	–2.575

[a]The models are explained in Fig. 1; see also the text.
[b]These values point out that w is not larger than the f value. The f value is $f(p_B - p_A, n - p_B)$ at the desired level of probability ($f_{0.95}$).

comparing the F-statistic $f(p_B - p_A, n - p_B)$ at the desired level of probability ($f_{0.95}$) with quotient w:

$$w = \frac{(SSE_A - SSE_B)\,(n - p_B)}{(p_B - p_A)SSE_B} \tag{3}$$

The f values are obtained from statistical tables (F distribution), $p_B - p_A$ (number of parameters added), and $n - p_B$ (degrees of freedom). Comparison of quotient w and the f value allows a discrimination among rival models that are convertible by the addition or removal of parameters *(38)*; that is if the quotient w is larger than the f value ($w - f$ value > 0), the choice of the more complex model is warranted—otherwise the simpler model should be preferred.

The results show in general a better fit when all the constants in the Fig. 1 model are taken into account (Table 2), as indicated by the *SSE* value. However, the increase in parameters implies that they are obtained with less accuracy, and if no significant difference exists, the simpler model should be preferred *(38)*. The *SSE* value comparison of all models (Table 2) clearly shows that CI, MI, MIPI, MISI, and MISPI should be preferred. Nevertheless, the $w - f$ value comparison of these five models (Table 3) proves that the CI should be preferred ($w - f$ value < 0). Furthermore the K_{iu}, K_{ip}, and K_{is} parameters are so large that they can be eliminated from models MISPI and MISI (Table 2). These results support the hypothesis that the parabolic inhibition and the formation of nonproductive complexes are not the principal constraints to cellulose hydrolysis.

Our results show that cellobiose is a strong competitive inhibitor of the activity of Cel7A with a K_{ic} of about 0.041 m*M*. The other kinetic constants are $K_m = 3.8$ m*M* and $k_{cat} = 2\ h^{-1}$ ($5.6 \times 10^{-4}\ s^{-1}$). Experimental results and fitted values assuming the CI model support this assumption and allow us to fit a curve to the experimental data on cellulose hydrolysis (Fig. 4). It is important to point out that the inhibition constant by cellobiose is in the same order of magnitude when compared with other results *(39,40)*. The inhibi-

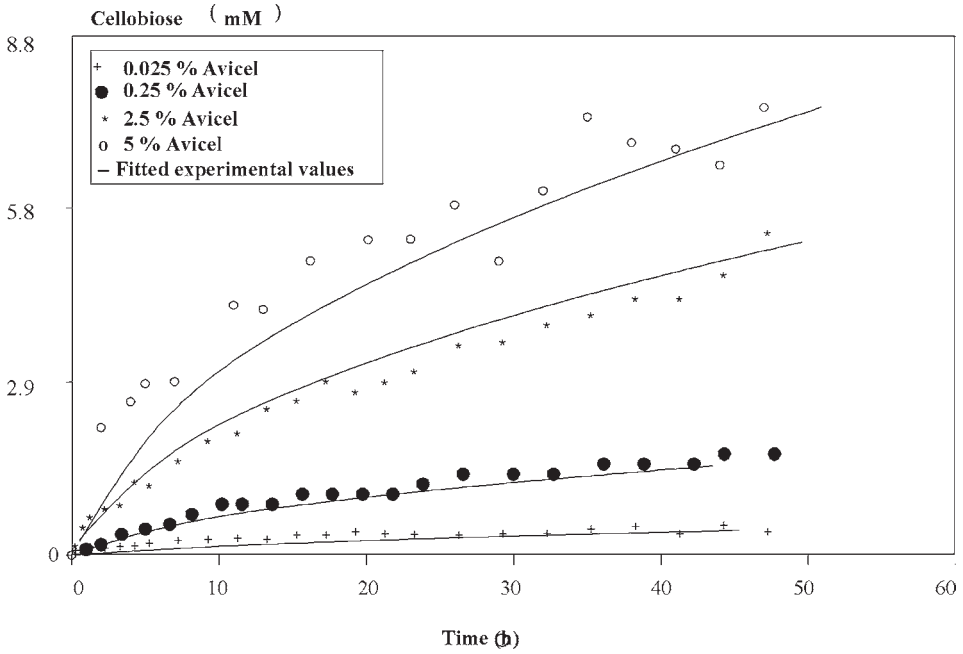

Fig. 4. Experimental points and fitted curves of cellulose hydrolysis with exoglucanase Cel7A (10 µg/mL). The model utilized in fitted curves was CI. The model with tabulated constants (Table 3) allowed fitting beyond the figure values of all [*S*]/[*E*] ratios up to 6.5 (563 experimental points).

tion constant is currently considered to be more effective (lower values) when compared with the values presented some years ago *(41)*.

*[S]/[*Et*] Ratio*

With these models it is possible to predict the hydrolysis of the cellulose, while the relationship between the substrate and the enzyme remains in agreement with Michaelis-Menten kinetic theory. The usual equations of enzymatic kinetics assume that the enzyme concentration is very much smaller than the substrate concentration. Hydrolysis augmentation related to an increase in enzyme concentration is not significant for [*S*]/[*E*] ratios of <6.5. The surface plot in Fig. 5 shows the three-dimensional shape of our data (interpolation of experimental points) using an SAS software package. The plane surface at the top of Fig. 5 suggests that the increase in enzyme concentration up to a certain value has no influence on the reaction product concentration, showing the economic importance of this ratio in the improvement of industrial applications of cellulases. In the enzymatic hydrolysis of cellulose by CBHs, the [*S*]/[*E*] ratios are especially important because the relative molecular mass of the enzyme ($M_r = 66 \times 10^3$) and the substrate have the same order of magnitude and therefore give a rapid saturation of hydrolysis sites.

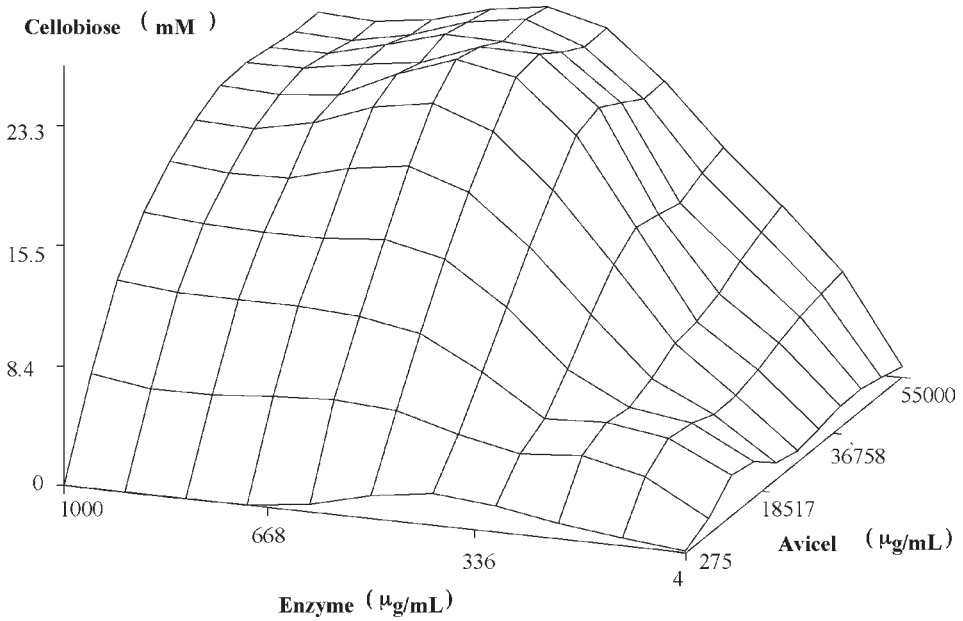

Fig. 5. Surface obtained with exoglucanase Cel7A from experimental results that allowed fitting variables of axes. The concentration of initial cellobiose is zero. The concentration of the product formed (cellobiose) corresponds to hydrolysis for 47 h. The top plan surface of the graphic shows that the increase in enzyme and substrate concentration upward of a certain value does not influence the concentration of the obtained product.

Conclusion

Discrimination among eight Michaelis-Menten models of inhibition by cellobiose and by the substrate showed that hydrolysis of cellulose follows a model of competitive inhibition with the following parameters: $K_m = 3.8$ mM, $K_{ic} = 0.041$ mM, $k_{cat} = 2\ h^{-1}$ ($5.6 \times 10^{-4}\ s^{-1}$). These constants were obtained with a large range of [S]/[Et] ratios and demonstrate that the Cel7A kinetic does not change with these different ratios within a considered range. The possibilities of uncompetitive, noncompetitive, parabolic, mixed linear inhibition, and a special case of substrate inhibition with the formation of nonproductive enzyme substrate complexes are not confirmed as the principal constraints in enzymatic cellulose hydrolysis. The Selwyn test has also pointed out that denaturation of the enzyme during the catalysis is not significant and cannot explain the rate decrease of the enzyme-catalyzed reaction.

Nomenclature

E = free enzyme
EI = enzyme inhibitor complex
EII = enzyme inhibitor complex for second inhibitor molecule
EIS = enzyme substrate inhibitor complex

ES = enzyme substrate complex (nonproductive)
Et = total enzyme
$f_{0.95}$ = point of Fp_A,p_B (F distribution) curve with area 0.95 (to its left)
k_{cat} = catalytic constant (h^{-1})
K_{ic} = competitive inhibition constant (mM)
K_{ip} = parabolic inhibition constant (mM)
K_{is} = substrate inhibition constant (mM)
K_{iu} = uncompetitive inhibition constant (mM)
K_m = Michaelis constant (mM)
n = experimental points
p_A, p_B = parameters
P = product (μg/mL) (cellobiose)
P_0 = initial product
P_t = product at time t (min)
R^2 = determination coefficient
S = substrate (%) (cellulose)
SSE = sum of squares error
t = time (min)
V_{max} = maximum velocity
w = quotient used to test significance of improvement of different models that are interconvertible by addition or elimination of parameters in comparison with F value

References

1. Ladisch, M. R. and Svarczkopf, J. A. (1991), *Bioresour. Technol.* **36,** 83–95.
2. Kurakake, M., Shirasawa, T., Ooshima, H., Converse, A. O., and Kato, J. (1995), *Appl. Biochem. Biotechnol.* **50,** 231–241.
3. Howell, J. A. and Mangat, M. (1978), *Biotechnol. Bioeng.* **20,** 847–863.
4. Nidetzky, B., Steiner, W., Hayn, M., and Esterbauer, H. (1993), *Bioresour. Technol.* **44,** 25–32.
5. Lee, Y.-H. and Fan, L. T. (1982), *Biotechnol. Bioeng.* **24,** 2383–2406.
6. Pereira, A. N. (1987), PhD thesis, Purdue University, West Lafayette, IN.
7. Kim, D. W., Kim, T. S., Jeong, Y. K., and Lee, J. K. (1992), *J. Ferment. Bioeng.* **73(6),** 461–466.
8. Nidetzky, B., Hayn, M., Macarron, R., and Steiner, W. (1993), *Biotechnol. Lett.* **15(1),** 71–76.
9. Eriksson, T., Karlsson, J., and Tjerneld, F. A. (2002), *Appl. Biochem. Biotechnol.* **101,** 41–60.
10. Howell, J. A. and Stuck, J. D. (1975), *Biotechnol. Bioeng.* **17,** 873–893.
11. Bader, J., Bellgardt, K.-H., Singh, A., Kumar, P. K. R., and Schügerl, K. (1992), *Bioprocess Eng.* **7,** 235–240.
12. Maglione, G., Russel, J. B., and Wilson, D. B. (1997), *Appl. Environ. Microbiol.* **63(2),** 665–669.
13. Schülein, M. J. (1997), *Biotechnology* **57(1–3),** 71–81.
14. Bezerra, R. M. (1995), PhD thesis, Universidade de Trás-os-Montes e Alto Douro, Vila Real, Portugal.
15. Hsu, T.-A. (1979), PhD thesis, Purdue University, West Lafayette, IN.
16. Lee, Y.-H. and Fan, L. T. (1983), *Biotechnol. Bioeng.* **25,** 939–966.
17. Beldman, G., Leeuwen, S.-V., Rombouts, F. M., and Voragen, F. G. J. (1985), *Biochem. J.* **146,** 301–308.

18. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem.* **193,** 265–275.
19. Wood, T. M. and Bhat, K. M. (1988), *Methods Enzymol.* **160,** 87–113.
20. Klyosov, A. A. and Rabinovitch, M. L. (1980), in *Enzyme Engineering Future Directions*, Wingard, L. B. Jr., Berezine, I. V., and Klyosov, A. A., eds., Plenum, New York, pp. 83–165.
21. Nidetzky, B., Steiner, W., and Claeyssens, M. (1994), *Biochem. J.* **303,** 817–823.
22. Väljamäe, P., Petterson, G., and Johansson, G. (2001), *Eur. J. Biochem.* **268,** 4520–4526.
23. Harjunpää, V., Teleman, A., Koivula, A., Ruohonen, L., Teeri, T. T., Teleman, O., and Drakenberg, T. (1998), *Eur. J. Biochem.* **240,** 584–591.
24. Segel, H. I. (1993), in *Enzyme Kinetics*, John Wiley & Sons, New York, pp. 18–89.
25. Fujii, M., Homma, T., Ooshima, K., and Taniguchi, M. (1991), *Appl. Biochem. Biotechnol.* **28/29,** 145–156.
26. Fernly, H. N. (1974), *Eur. Biochem. J.* **43,** 377, 378.
27. Yun, S.-L. and Suelter, C. H. (1977), *Biochim. Biophys. Acta* **480,** 1–13.
28. Mangat, M. N. and Howell, J. A. (1978), *Food Pharm. Bioeng.* **74(172),** 77–81.
29. Ladisch, M. R., Lin, K. W., Voloch, M., and Tsao, G. T. (1983), *Enzyme Microb. Technol.* **5(2),** 81–102.
30. Ghose, T. K., Roychoudhury, P. K., and Ghosh, P. (1984), *Biotechnol. Bioeng.* **26,** 377–381.
31. Golovchenko, N. P., Kataeva, I. A., and Akimenko, V. K. (1992), *Enzyme Microb. Technol.* **14,** 327–331.
32. Orgeret, C., Seillier, E., Gautier, C., Defaye, J., and Driguez, H. (1992), *Carbohydr. Res.* **224,** 29–40.
33. Lee, Y.-H. and Fan, L. T. (1983), *Biotechnol. Bioeng.* **25,** 939–966.
34. Holtzapple, M., Cognata, M., Shu, Y., and Hendrickson, C. (1990), *Biotechnol. Bioeng.* **36,** 275–287.
35. Duggleby, R. G. (1995), *Methods Enzymol.* **249,** 61–90.
36. Selwyn, M. J. (1965), *Biochim. Biophys. Acta* **105,** 193–195.
37. Ralston, M. L. and Jennrich, R. I. (1978), *Technometrics* **20(1),** 7–14.
38. Mannervik, B. (1982), *Methods Enzymol.* **87C,** 370–391.
39. Igarashi, K., Samejima, M., and Eriksson K.-E. L. (1998), *Eur. J. Biochem.* **253,** 101–106.
40. Henriksson, H., Ståhlberg, J., Isaksson, R., and Pettersson, G. (1996), *FEBS Lett.* **390,** 339–344.
41. Ladisch, M. R., Hong, J., Voloch, M., and Tsao, G. T. (1981), in *Trends in the Biology of Fermentations for Fuels and Chemicals*, Hollaender, A., Rabson, R., Pietro, R., and Wolfe, eds., Plenum, New York, pp. 55–83.