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Simultaneous Ethanol and Cellobiose Inhibition of Cellulose Hydrolysis Studied With Integrated Equations Assuming Constant or Variable Substrate Concentration

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Abstract

The integrated forms of the Michaelis-Menten equation assuming variable substrate (depletion) or constant substrate concentration were used to study the effect of the simultaneous presence of two exoglucanase Cel7A inhibitors (cellobiose and ethanol) on the kinetics of cellulose hydrolysis. The kinetic parameters obtained, assuming constant substrate (K_m = 21 m*M*, K_{ic} = 0.035 m*M*; $K_{icl} = 1.5 \times 10^{15}$ m*M*; k_{cat} = 12 h^{-1}) or assuming variable substrate (K_m = 16 m*M*, K_{ic} = 0.037 m*M*; $K_{icl} = 5.8 \times 10^{14}$ m*M*; k_{cat} = 9 h^{-1}), showed a good similarity between these two alternative methodologies and pointed out that both ethanol and cellobiose are competitive inhibitors. Nevertheless, ethanol is a very weak inhibitor, as shown by the large value estimated for the kinetic constant K_{icl}. In addition, assuming different concentrations of initial accessible substrate present in the reaction, both inhibition and velocity constants are at the same order of magnitude, which is consistent with the obtained values. The possibility of using this kind of methodology to determine kinetic constants in general kinetic studies is discussed, and several integrated equations of different Michaelis-Menten kinetic models are presented. Also examined is the possibility of determining inhibition constants without knowledge of the true accessible substrate concentration.

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Index Entries: Cellulase kinetics; ethanol inhibition; exoglucanase Cel7A; integrated Michaelis-Menten equations.

Introduction

Cellobiose inhibits cellulases 14 times more than glucose *(1)* and is considered a powerful inhibitor of cellulases that regulate cellulose saccharification *(2–5)*. This type of inhibition has been classified in many different ways. The apparent confusion results mostly from both the difficulty of conducting conclusive experiments to show the type of inhibition in a heterogeneous reaction environment (cellulose is an insoluble substrate) and the assumption that the cellulolytic system consists of a single enzyme *(6)*. Despite previous results that pointed out different types of inhibition according to the substrate/enzyme ratio *(6)*, we have recently obtained a competitive inhibition model with both a *Trichoderma reesei* cellulolytic system and purified Cel7A exoglucanase that is applicable to a large range of substrate/enzyme ratios *(7)*.

Ethanol inhibition is much less potent than cellobiose inhibition but presents an important problem in the simultaneous saccharification and fermentation of biomass *(8)*. Holtzaple et al. *(9)* concluded that the effect of ethanol inhibition is six times less potent than that of glucose inhibition. Cellulase inhibition by ethanol is considered noncompetitive for ethanol concentrations less than 4 *M (9,10)*. Nevertheless, purified Cel7A exhibits a very weak competitive inhibition *(7)*, and β-glucanase is not affected by low ethanol concentrations *(11,12)*.

Despite some difficulties related to the change of kinetic conditions during the reaction, integrated Michaelis-Menten equations can make an important contribution to enzymatic kinetic studies *(13–15)*. The application of nonlinear regression with integrated Michaelis-Menten equations has advantages when compared with linear methods, because more kinetic constants can be determined by the same experimental results. It is also possible to determine the kinetic parameters with fewer statistical errors and without constants transformations *(16)*. The possibility of determining product inhibition constants without the addition of this product inhibitor, or the possibility of determining kinetics with more than one inhibitor, is of special importance *(17,18)*. In addition, the possibility of determining the kinetic constants without knowledge of the true substrate concentration, and the possibility of substrate quantification by nonlinear fitting of a progress curve to the integrated Michaelis-Menten equation, have already been reported (*19–21*).

The present work is a further development of previous studies *(7,22)* into the kinetic of cellulose hydrolysis using integrated Michaelis-Menten equations. Our main objectives were (1) to surpass the difficulties of not knowing the true accessible substrate concentration and (2) to study the kinetic of an additional inhibitor (ethanol) in the presence of a mandatory product inhibitor (cellobiose). In this article, we also discuss the use of this methodology for the study and understanding of general enzyme inhibition.

Materials and Methods

Enzyme Preparation and Chemicals

Celluclast 1,5 L, a commercial cellulase preparation from *T. 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

Purification of *T. reesei* exoglucanase Cel7A from Celluclast 1,5 L (Novo Nordisk A/S) was accomplished according to Beldman et al. *(23)* and Pereira *(2)* with modifications as previously described *(22)*. Briefly, dialylfiltrated crude enzyme was submitted to gel filtration chromatography with Bio-Gel P10 medium (Bio-Rad 150-1040) and DEAE Bio-Gel A (Bio-Rad 153-0740) anion-exchange chromatography. Finally, affinity chromatography with Avicel resulted in an exoglucanase Cel7A–enriched fraction, as indicated by the ratio of Avicelase to carboxymethylcellulase (CMCase). The homogeneity of this fraction was confirmed, and a single band appeared in polyacrylamide gels (sodium dodecyl sulfate polyacrylamide gel electrophoresis). All kinetic studies were performed with this purified fraction.

Enzyme Activities

The enzyme activities (CMCase, Avicelase, and aryl-β-glucosidase) were measured according to IUPAC-Biotechnology Commission procedures. The reducing sugars released were determined by dinitrosalicylic acid or the Somogyi-Nelson method, using cellobiose as a standard *(22)*.

Kinetic Assays

Kinetic studies were performed at 40°C in 50 m*M* citrate buffer, pH 4.8, with 0.01% sodium azide and monitored for 47 h (22 sampling points for each run). Different experiments were carried out with four concentrations of substrate (Avicel) (5.0, 2.5, 0.25, and 0.025% [w/v], which were equivalent to 154.2, 77.1, 7.7, and 0.77 m*M* potential cellobiose); six concentrations of Cel7A (4, 10, 42, 100, 417, and 1000 µg/mL); and four concentrations of ethanol (0.0, 0.43, 0.86, and 1.72 *M*). Identical experiments with added initial cellobiose (7.5 and 15.0 m*M*) without ethanol were also realized at the 5.0 and 2.5% (w/v) substrate concentrations and exoglucanase Cel7A concentrations of 100 and 1000 µg/mL.

For each assay, a complete progress curve was analyzed. To fit the experimental values with integrated Michaelis-Menten equations, substrate/enzyme ratios ([*S*] [µg/mL]/[*E*] [µg/mL]) less than 6.5 were not considered, as previously explained *(22)*. Briefly, in our experimental conditions, [*S*]/[*E*] ratios less than 6.5 are in contradiction with usual assump-

tions of enzymatic kinetics because enzyme concentration should be much smaller than substrate concentration *(7,24)*.

The kinetic parameters from the integrated Michaelis-Menten equations were evaluated from nonlinear least square (DUD method). The DUD method is a derivative-free Gauss-Newton algorithm that is particularly well suited when the calculus of derivatives is not an easy task *(7,22,24,25)*.

Results and Discussion

Theoretical Framework

Linear mixed inhibition includes all of the common types of inhibition and will therefore be taken as a general case (Fig. 1), although some inhibition constants can tend to infinity, meaning that they are irrelevant. This methodology has already been explained in previous works *(7,22,26)* and can be described by the following rate equation (*see* Nomenclature and Fig. 1 for an explanation of the terms):

$$v = \frac{V[So]}{K_m(1 + [I]/K_{ic}) + [So](1 + [I]/K_{iu})}$$

It is assumed that the inhibitor concentration $[I]$ is equal to the product concentration $[P]$ plus the ethanol concentration $[A]$, as previously explained *(7)*. Thus, when the ethanol concentration is not present $[I] = [P]$. Therefore, in a similar way, with competitive inhibition we can define an ethanol inhibition constant as K_{icl} and a cellobiose inhibition constant as K_{ic} or in the same mode a K_{iul} and a K_{iu} to a noncompetitive inhibition *(7,27)*. The obtained equation assuming constant substrate concentration *(7)* is as follows (*see* Fig. 1 and Nomenclature):

$$v = \frac{dP}{dt} = \frac{1}{K_m/(V[So])(1 + [P]/K_{ic} + [A]/K_{icl}) + 1/V(1 + [P]/K_{iu} + [A]/K_{iul})}$$

$$t = \frac{1}{V}\left\{\left(\frac{K_m}{[So]} + \frac{K_m[A]}{[So]K_{icl}} + \frac{[A]}{K_{iul}} + 1\right)([Pt] - [Po]) + \left(\frac{K_m}{2[So]K_{ic}} + \frac{1}{2K_{iu}}\right)([Pt]^2 - [Po]^2)\right\}$$

in which Pt is the concentration of product at the time t and the subscript o; thus, So and Po means initial concentration at $t = 0$. Hence, $[P] = [Po] + [Pt]$, if there is no initial presence of inhibitor $[P] = [Pt]$.

The equation can also be integrated assuming that the substrate concentration decreases with time ($[So] = [St] + [Pt]$)

$$-V\int_o^t dt = \left(-\frac{K_m}{K_{ic}} + 1 + \frac{[So]}{K_{iu}} + \frac{[Po]}{K_{iu}} + \frac{[A]}{K_{iul}}\right)\int_{[So]}^{[St]} d[S] +$$

$$\left(K_m + \frac{K_m[So]}{K_{ic}} + \frac{K_m[Po]}{K_{ic}} + \frac{K_m[A]}{K_{icl}}\right)\int_{[So]}^{[St]} \frac{1}{St}\, d[S] + \left(-\frac{1}{K_{iu}}\right)\int_{[So]}^{[St]} Std[S]$$

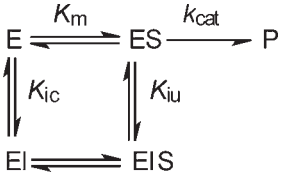

Fig. 1. Mixed linear (total) inhibition model (MI) in which E = enzyme; ES = enzyme substrate complex; EIS = enzyme substrate inhibitor complex; EI = enzyme inhibitor complex; P = product; K_m = Michaelis-Menten constant; K_{ic}, K_{iu} = inhibition constants; and k_{cat} = rate constant. A model without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

Table 1
Integrated Michaelis-Menten Equations Obtained
by Simplification of Mixed Linear Inhibition Equation Assuming $[So] = [St] + [Pt]$

Kinetic model[a]	Integrated equations
WI	$t = -\frac{1}{V}\left[K_m \ln \frac{St}{So} + (St - So)\right]$
CI	$t = -\frac{1}{V}\left[K_m \left(\frac{So}{K_{ic}} + \frac{Po}{K_{ic}} + 1\right) \ln \frac{St}{So} + \left(1 - \frac{K_m}{K_{ic}}\right)(St - So)\right]$
NCI	$t = -\frac{1}{V}\left[K_m \left(\frac{So}{K_{iu}} + \frac{Po}{K_{iu}} + 1\right) \ln \frac{St}{So} + \left(1 - \frac{K_m}{K_{iu}} + \frac{Po}{K_{iu}} + \frac{So}{K_{iu}}\right)(St - So) - \frac{1}{2K_{iu}}(St^2 - So^2)\right]$
UCI	$t = -\frac{1}{V}\left[K_m \ln \frac{St}{So} + \left(1 + \frac{So}{K_{iu}} + \frac{Po}{K_{iu}}\right)(St - So) + \left(-\frac{1}{2K_{iu}}\right)(St^2 - So^2)\right]$

[a]WI, without inhibition; CI, competitive linear inhibition; NCI, noncompetitive linear inhibition ($K_{ic} = K_{iu}$); UCI, uncompetitive inhibition.

to obtain the following equation:

$$t = -\frac{1}{V}\{K_m\left(\frac{[So]}{K_{ic}} + \frac{[Po]}{K_{ic}} + \frac{[A]}{K_{icl}} + 1\right)\ln\frac{[St]}{[So]} + \left(1 - \frac{K_m}{K_{ic}} + \frac{[So]}{K_{iu}} + \frac{[Po]}{K_{iu}} + \frac{[A]}{K_{iul}}\right)([St] - [So]) + \left(-\frac{1}{2K_{iu}}\right)([St^2] - [So^2])\}$$

It should be pointed out that these equations can also be obtained by equivalent integrations but with a different mathematical expression *(27,28)*.

The last equation (mixed linear inhibition) can be simplified, giving rise to models with fewer constants. In Table 1, to simplify these equations the absence of the second inhibitor (in this case ethanol) was assumed.

Discrimination and Obtained Constants

This kinetic investigation consists of two parts: discrimination between available models and parameter estimation (Tables 2 and 3).

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

	WI	*CI*	*NCI*	*UCI*	*MI*
K_m (m*M*)	10	16	1050	10	16
K_{ic} (m*M*)		0.037	0.047		0.037
K_{icl} (*m*M)		5.8×10^{14}	2.4×10^{9}		1.2×10^{18}
K_{iu} (*m*M)			0.047	6.5×10^{13}	1.3×10^{19}
K_{iul} (m*M*)			2.4×10^{9}	6.1×10^{12}	1.9×10^{22}
k_{cat} (h^{-1})	2	9	489	2	9
SSE	366,524	171,832	177,462	366,524	171,716
R^2	0.38	0.71	0.70	0.38	0.71
p	2	4	4	4	6
n	1043	1043	1043	1043	1043

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

As previously demonstrated *(29)* 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 sum of squares error (SSE) value should be regarded as giving the "best" fit and is a measure of the goodness of fit of the mathematical model to the data set *(29)*.

$$w = \frac{(\mathrm{SS\,E}_A - \mathrm{SSE}_B)(n - p_B)}{(p_B - p_A)\mathrm{SSE}_B}$$

When $w - f$-value > 0, the more complex model should be preferred; otherwise a simpler model can be applied. The *f*-values are obtained from statistical tables (F distribution, $f(p_B - p_A, n - p_B)$) at the desired level of probability ($f_{0.95}$) when $p_B - p_A$ (number of parameters added) and $n - p_B$ (degrees of freedom) are known for these models (Tables 2 and 3).

By studying the values presented in Table 2, one can conclude that competitive inhibition (CI), noncompetitive inhibition (NCI), and uncompetitive inhibition (UCI) have the same parameters; thus, it is more than enough to compare the SSE values. Comparison reveals that the CI model is better than any other with the same number of parameters and with the lowest SSE value. Thus, it is necessary to compare only the $w - f_{0.95}$ value to the without inhibition (WI) vs CI model and the CI vs linear mixed inhibition (MI) model (Table 3). The $w - f_{0.95}$ comparison between models (Table 3) shows that CI should be preferred when compared with the WI or MI models. The effect of cellobiose and ethanol in the cellulose hydrolysis with Cel7A is shown in Fig. 2. Fitted curves were obtained with constants of CI model (Table 2).

When investigating cellulose hydrolysis, several researchers have assumed that the substrate accessible to the enzyme is the existent substrate and have not considered the existence of the accessibility factor *(28)*. Kleman-Leyer and Kirk *(30)* suggested that the cellulose structure is

Table 3
Summary of $w - f_{0.95}$ Values (*see* Text) in Assays With Cel7A With Ethanol (Assuming Variable Substrate)[a]

Models A/B	SSE_A	SSE_B	n	p_A	p_B	w	$p_B - p_A$	$n - p_B$	$f_{0.95}$	$w - f_{0.95}$
WI/CI	366524	171832	1043	2	4	589.36	2	1039	3.00	586.36
WI/NCI	366524	177462	1043	2	4	553.46	2	1039	3.00	550.46
WI/UCI	366524	366524	1043	2	4	0.0	2	1039	3.00	–3.00[b]
WI/MI	366524	171716	1043	2	6	294.1	4	1037	2.37	291.74
CI/NCI	171832	177462	1043	4	4					[c]
CI/UCI	171832	366524	1043	4	4					[c]
CI/MI	171832	171716	1043	4	6	–0.35	2	1037	3.00	–2.65[b]
NCI/UCI	177462	366524	1043	4	4					[c]
NCI/MI	177462	171716	1043	4	6	17.35	2	1037	3.00	14.35
UCI/MI	366524	171716	1043	4	6	588.23	2	1037	3.00	585.23

[a]The models are explained in Fig. 1; also *see* 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 ($f_{0.95}$).
[c]p_A is equal to p_B (to compare these models *see* SSE).

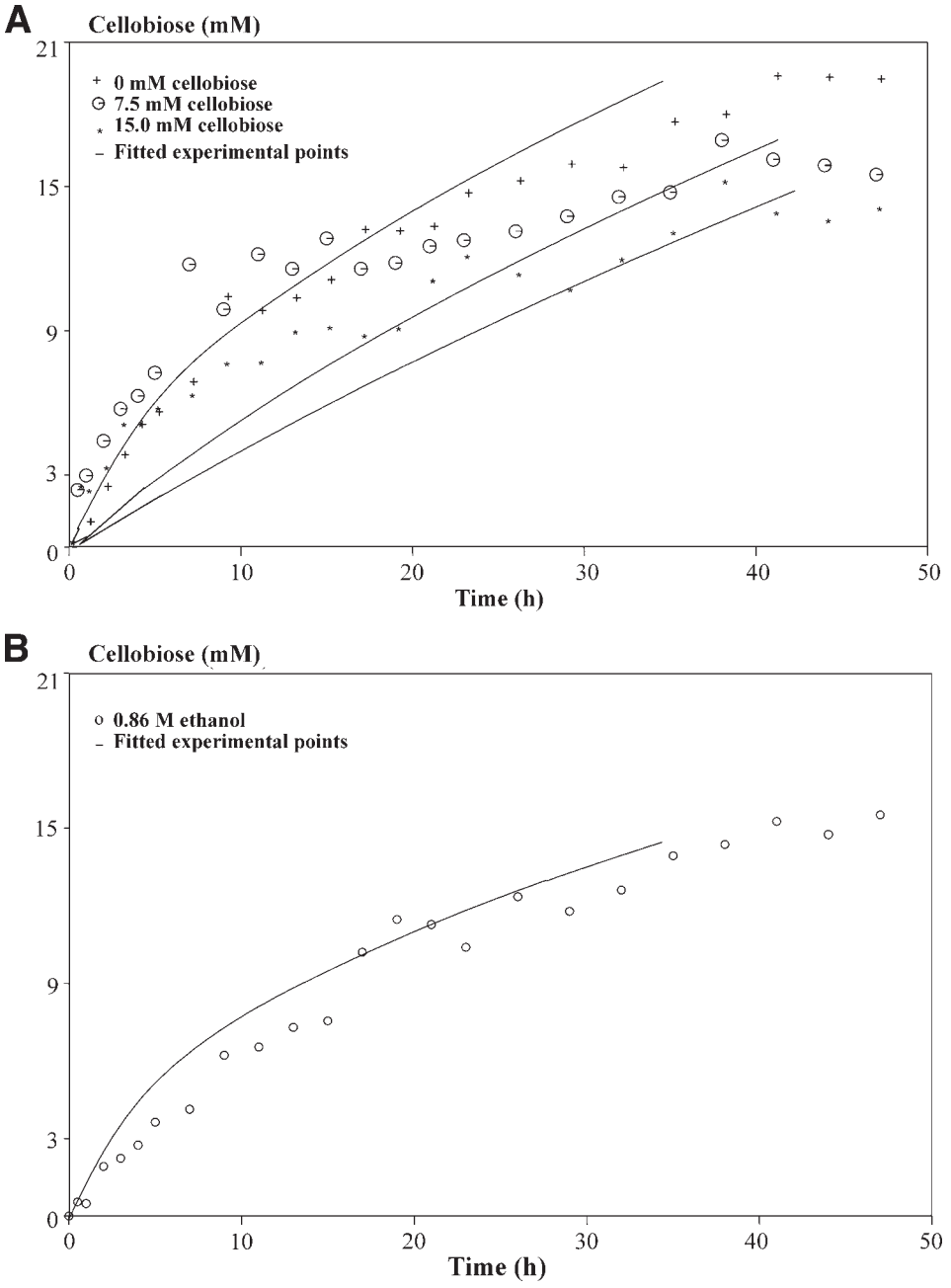

Fig. 2. Experimental points and fitted curves of cellulose hydrolysis with Cel7A exoglucanase (1000 µg/mL) and 2.5% (w/v) Avicel. The model utilized in fitted curves was CI according to tabulated constants (Table 2): (**A**) cellulose time-course hydrolysis in presence of different cellobiose concentrations; (**B**) cellulose time-course hydrolysis in simultaneous presence of cellobiose (product of reaction) and ethanol.

Table 4
Summary of Obtained Constants in Assays With Fraction Cel7A Assuming Constant or Variable Substrate

	K_m (m*M*)	K_{ic} (m*M*)	K_{icl} (m*M*)	k_{cat} (h^{-1})
Assuming constant substrate	21.1 (2.1[a]; 1.1[b])	0.035 (0.035[a]; 0.035[b])	1.5×10^{15} (6.5×10^{16}[a]; 6.3×10^{14}[b])	12 (12[a]; 12[b])
Assuming variable substrate	15.8 (7.9[c])	0.037 (0.042[c])	5.8×10^{14} (2.3×10^{12}[c])	9 (9[c])

[a]Assuming 10% of substrate accessible ($\phi = 0.1$).
[b]Assuming 5% of substrate accessible ($\phi = 0.05$).
[c]Assuming 50% of substrate accessible ($\phi = 0.5$).

temporarily modified by the action of cellulases, allowing the enzyme molecules to flow into the cellulose structure. Thus, all potential substrate is accessible to the enzyme action. Gusakov and Sinitsyn *(6)* consider that the accessible substrate is proportional to all substrate present in the reaction medium. However, our results support the hypothesis that the accessible factor does not affect the obtained inhibition constants when the enzyme is exoglucanase Cel7A. The reason for this is the constant proportion between the accessible substrate and total substrate in the reaction medium.

However, it is difficult to determine the effective cellulose concentration in the heterogeneous reactions catalyzed with cellulases. Thus, let us define the total existent substrate (S) and the accessible substrate to the enzyme (S'') according to the following equation, in which ϕ is an accessibility factor that can vary between 0 and 1:

$$S\phi = S''$$

If the substrate is affected by a value ϕ, none of the inhibition constants are altered, as was explained previously *(25,31,32)*.

In general, the same values for the kinetic constants (Table 4) were obtained assuming a constant (7) or variable ($[So] = [St] + [Pt]$) substrate concentration. The equations that consider the substrate constant and variable are different and therefore reinforce the theory that the ϕ value is annulled and, especially, that the inhibitions constants are not affected by the lack of accurate knowledge of the true substrate concentration. In addition, Table 4 shows the kinetic constants obtained when the accessible substrate is only 50, 10, or 5% (*see* notes to Table 4). With the models that consider the concentration of the substrate as variable only one division was possible (50% of initial substrate). When the division was by a greater number, the DUD method did not converge, owing to the greater number of baseless values, because the product surpasses the concentration of substrate initially present.

In the models when the initial substrate value was divided by the respective factor ϕ, no changes of considered kinetic constants are obtained. Only the K_m is affected by the some proportion assumed by the accessible

factor ϕ. This verification supports the hypothesis that these values are real constants, as the Michaelis-Menten theory assumes. This is foreseen by the results previously explained *(25,31,32)* once the kinetic constants are not influenced by the fact that real substrate concentration is unknown. The results support the hypothesis that the Michaelis-Menten equations can fit the progress curve of cellulose hydrolysis.

The values obtained in Table 4 indicate a very weak ethanol inhibition, as previously pointed out *(7)*. In fact, Cel7A, in contrast to the crude cellulase complex, is almost not inhibited by ethanol. Thus, an apparent contradiction exists because exoglucanase Cel7A dominates the cellulase complex of *T. reesei* and represents the cellulolytic workhorse *(7,24)*.

Because the enzymes and in general the proteins create colloidal dispersions, for a number of years it was believed that Langmuir adsorption must occur, and many times this was assumed in the kinetic of cellulose hydrolysis *(33)*. The Langmuir *(34)* model was considered for a long time as an alternative to the Michaelis-Menten model. In the present study, the adsorption of cellulase was considered to be similar to what happens with soluble substrate and enzymes, studied from the perspective of the Michaelis-Menten kinetic. The results obtained confirm the possibility of modeling cellulose hydrolysis by the Michaelis-Menten kinetic theory.

The possibility of utilizing this methodology to determine kinetic constants in general kinetic studies and the possibility of developing a spectrophotometer software to indicate "on line" the type of inhibition and kinetic constants seems to us important to point out. In fact, an integrated equation allows the determination of accurate initial velocities even when the reaction product is an inhibitor, and this determination should be the first choice among rival kinetic models. This methodology has already been used with alkaline phosphatase, a typical Michaelis-Menten kinetic with two different inhibitors simultaneously present and the constants have been compared with traditional methods *(26)*. Furthermore, the methodology can be used with the experimental points obtained from the progress curve *(7,22)* or with only limited data points usually utilized to determine initial velocities *(26)*.

Conclusion

Theoretical considerations about the integrated equations in the study of enzyme kinetics, especially in cellulose hydrolysis, point out the importance of the Michaelis-Menten kinetic to explain cellulose hydrolysis. In general, the same values for the kinetic constants were obtained from models that assume variable substrate as well as constant substrate. Previous results suggest that the decrease in substrate does not affect Avicel hydrolysis and that the inhibition product is probably of major importance. The results also suggest the superiority of competitive inhibition compared with noncompetitive inhibition. Finally, a heterogeneous reaction, such as cellulose hydrolysis, can be studied by Michaelis-Menten equations, and

the kinetic constants (except K_m) are not influenced by a lack of knowledge of the true substrate concentration.

Nomenclature

A = ethanol
E = free enzyme
$f_{0.95}$ = point of Fpa,pb (F distribution) curve with area 0.95 (to its right)
I = all inhibitors
k_{cat} = catalytic constant (h^{-1})
K_{ic} = competitive inhibition constant (m*M*) to cellobiose
K_{icl} = competitive inhibition constant (m*M*) to ethanol
K_{iu} = uncompetitive inhibition constant (m*M*) to cellobiose
K_{iul} = uncompetitive inhibition constant (m*M*) to ethanol
K_m = Michaelis constant (m*M*)
n = experimental points
P = reaction product (cellobiose)
p_A, p_B = parameters
Po = initial product
Pt = product at time t (min)
S = substrate
t = time (min)
V_{max} = maximum velocity
w = quotient used to test significance of improvement of different models interconvertible by addition or elimination of parameters by comparison of *F*-value

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