Appl Biochem Biotechnol (2011) 165:178–189
DOI 10.1007/s12010-011-9242-y

Cellulose Hydrolysis by Cellobiohydrolase Cel7A Shows Mixed Hyperbolic Product Inhibition

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Received: 13 January 2011 / Accepted: 4 April 2011 /
Published online: 16 April 2011

Abstract In order to establish which are the contribution of linear (total), hyperbolic (partial) or parabolic inhibitions by cellobiose, and also a special case of substrate inhibition, the kinetics of cellobiohydrolase Cel7A obtained from *Trichoderma reesei* was investigated. Values of kinetic parameters were estimated employing integrated forms of Michaelis–Menten equations through the use of non-linear regression, and criteria for selecting inhibition models are discussed. With cellobiose added at the beginning of the reaction, it was found that cellulose hydrolysis follows a kinetic model, which takes into account a mixed hyperbolic inhibition, by cellobiose with the following parameter values: K_m 5.0 mM, K_{ic} 0.029 mM, K_{iu} 1.1 mM, k_{cat} 3.6 h^{-1} and $k_{cat'}$ 0.2 h^{-1}. Cellulose hydrolysis without initial cellobiose added also follows the same inhibition model with similar values (4.7, 0.029 and 1.5 mM and 3.2 and 0.2 h^{-1}, respectively). According to Akaike information criterion, more complex models that take into account substrate and parabolic inhibitions do not increase the modulation performance of cellulose hydrolysis.

Keywords Cellulase kinetics · Cellobiohydrolase Cel7A · Integrated Michaelis–Menten equations · Hyperbolic, parabolic and substrate inhibitions · Diagnosis of enzyme inhibition

Nomenclature

E	Free enzyme
EI	Enzyme inhibitor complex
EIS	Enzyme substrate inhibitor complex
ES	Enzyme substrate complex
k_{cat}	Catalytic constant (per hour) to the breakdown of the ES complex

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$k_{cat'}$	Catalytic constant (per hour) to the breakdown of the ESI complex
K_{ic}	Competitive inhibition constant (millimolar) to cellobiose
K_{iu}	Uncompetitive inhibition constant (millimolar) to cellobiose
K_{ip}	Parabolic inhibition constant (millimolar) to cellobiose
K_m	Michaelis constant (millimolar)
n	Experimental points
P	Reaction product (cellobiose)
p_A, p_B	Parameters
Po	Initial Product
Pt	Product at time t (minutes)
S	Substrate
SSE	Sum of squares error
t	Time (minutes)
V_{max}	Maximum velocity

Introduction

The accurate determination of the kinetic properties of enzymes involved in cellulose hydrolysis can improve the use of lignocellulose raw materials. The Michaelis–Menten integrated equation traditionally has great importance in the research of cellulose hydrolysis and other biopolymers mainly because the reliable estimation of initial rate was experimentally difficult when strong product inhibition occurs [1, 2].

The generalized determination of initial velocities by a tangent to the product time curve is theoretically incorrect mainly when the product of reaction is a potent inhibitor. In these situations, the traditional methods to determine the initial velocity are strongly affected being the initial velocity determined obviously inferior to the real value. The principal advantages of integrated Michaelis–Menten equations are (a) the need to differentiate the data (i.e., to measure rates) is eliminated; (b) more information can be obtained from each experiment saving both time and materials; (c) the initial addition of end-product inhibitor can be eliminated and K_i may be simultaneously evaluated under conditions which do not require the separate addition of the reaction products; (d) enzymes are submitted to the action of reaction products with correct stereochemistry; and (e) the possibility to predict initial substrate concentration and to determine kinetic constants without the knowledge of substrate concentration [3–5]. In fact, Michaelis–Menten equations needs correct knowledge of real substrate concentration which should be always much greater than enzyme concentration [6]. However, integrated Michaelis–Menten equations overcome this limitation [3]. The reason for this is the constant proportion between accessible substrate and total substrate in the reaction medium [7, 8]. The most severely practical limitation of progress curve analysis occurs when the enzyme shows a loss of activity under the chosen assay conditions. Nevertheless, previous results show that the deactivation of Cel7A is not a constraint to the process [8].

Table 1 shows the main innovative characteristics of the models developed to understand cellulose hydrolysis. The main reason for the rate retarding effect of cellulose hydrolysis is still under discussion. It has been assumed to be related with denaturation or the transformation of substrate into a less digestible form even though the product inhibition has more scientific support. The type of product inhibition exhibited by cellulases is controversial. Uncompetitive inhibition was suggested by Lee and Fan [13], but competitive and non-competitive inhibitions are the most readily accepted forms of

Table 1 Innovative approaches in models of enzymatic cellulose hydrolysis

Reference	Innovative approaches
Huang [9]	First model to point out that Langmuir adsorption can explain cellulose enzymatic catalyze
Suga et al. [10]	Suggested that it is essential to consider the inhibition of cellulases by cellobiose in spite of the complexity of the equations obtained
Howell and Mangat [11]	Resuspended substrate (previously hydrolyzed) with new enzyme, suggesting enzyme denaturation
Ryu et al. [12]	Demonstrated that with low enzyme concentrations it is possible to ignore the Langmuir adsorption
Lee and Fan [13]	Pointed out that there are two fundamental aspects in cellulose hydrolysis: superficial area and crystallinity
Ohmine et al. [14]	Selected empirically based models because Michaelis–Menten equations did not predict the experimental results
Holtzapple et al. [15]	Proposed an "insoluble substrate equivalent" of the Michaelis–Menten model by the inclusion of parameters to account for adsorption and substrate available to enzyme
Fugii et al. [16]	Considered the possibility to assume constant substrate concentration during the reaction
Bader et al. [17]	The adsorption of cellulases to the substrate is regulated by the law of mass action and is similar to Michaelis kinetics in explaining the binding of enzyme to substrate.
Gusakov and Sinetsyn [7]	Concluded that the inhibition type changes with enzyme and substrate concentration
Converse and Optekar [18]	Pointed out the existence of co-operative effect, competitive adsorption and identified two types of substrate for cellobiohydrolases and endoglucanases
Sattler et al. [19]; Nidetzky et al. [20]	Pointed out the existence of two substrates types (amorphous and crystalline) and synergism effect
Väljamäe et al. [21]	Assumed fractal kinetics (reactants are spatially constrained originating non-uniformly mixed reaction species)
Zhang and Lynd [22]	Assumed the action of different enzymes (cellobiohydrolase I, cellobiohydrolase II, and endoglucanase I) and substrate parameters (degree of polymerization and fraction of accessible bonds)

inhibition [23]. Parabolic inhibition was initially considered by Pereira [24] and the possibility of some enzyme-cellulose complexes which are productive and others which are non-productive were also considered [20, 25]. In such circumstances, the need to develop systematic kinetic models and the necessity to discriminate these different models is obvious [26].

Most of the mechanistic models that describe cellulose hydrolysis in the literature used the Michaelis–Menten or Langmuir theories in spite of their constants could be interpreted differently. The Langmuir equation (as a relationship between the concentration of a compound adsorbing to binding sites and the fractional occupancy of the binding sites) is equivalent to the Hill equation [27]. Also, the Hill equation can be assumed as a development of Michaelis–Menten equation [28]. Already Ryu et al. [12] demonstrated that with low enzyme concentration, it is possible to ignore the Langmuir adsorption and the kinetics obey to conventional Michaelis–Menten kinetics. Other models that do not assume Michaelis–Menten or related theories are empirically based models may have interest in cellulose hydrolysis prediction but cannot explain its theoretical fundaments.

In this work, we investigate the nature of cellobiose inhibition assuming several inhibition types: competitive, non-competitive, uncompetitive, parabolic, hyperbolic inhibition and also the possibility of some enzyme-cellulose complexes, being productive and others being non-productive (special case of substrate inhibition). The fit of experimental data, assuming hyperbolic (partial) inhibition is worth noting because this hypothesis had been scarcely investigated [29]. The feasibility of using integrated equations in general kinetics and, particularly in biopolymers hydrolysis studies, may provide a more appropriate methodology to determine kinetic constants.

Material and Methods

Enzyme Preparation and Chemicals

Celluclast 1.5 L, a commercial cellulase preparation from *Trichoderma reesei*, was kindly provided by Novo Nordisk A/S (Copenhagen, Denmark) through its Portuguese representative (Meller and Essink).

Avicel, carboxymethylcellulose sodium salt, cellobiose and *p*-nitrophenyl-β-D-glucopyranoside were purchased from Merck. Other reagents used in the present study were of analytical grade.

Cellobiohydrolase Cel7A Purification

Purification of *T. reesei* cellobiohydrolase Cel7A (EC 3.2.1.91) from Celluclast 1.5 L (Novo Nordisk A/S Copenhagen Denmark) was accomplished according to Bezerra and Dias [26]. Briefly, initial gel filtration chromatography with Bio-Gel P10 was performed with 50 mM phosphate buffer, pH 7.0, with sodium azide 0.01% (*w*/*v*; 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 cut-off membrane and then submitted to DEAE Bio-Gel A (BioRad) 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 mM 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 re-equilibrated in 50 mM 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 sodium hydroxide 0.02% (*w*/*v*). The whole process was carried out at 4 °C. The amount of protein in the pooled fractions was determined by the Lowry method [30] 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 sulphate/polyacrilamide gel electrophoresis on a gradient 7% to 11% polyacrilamide gel was applied to check enzyme purity using the Laemmli discontinuous buffer system.

Enzyme Activities

The enzymatic activities (CMCase, Avicelase and aril-β-glucosidase) were measured according to the IUPAC-Biotechnology Commission procedures. The reducing sugars

released were determined by dinitrosalicylic acid or the Somogyi–Nelson method, using cellobiose as a standard [31].

Kinetic Assays

The kinetic studies were performed as explained previously [8, 26, 32]. Briefly, kinetic studies are performed at 40 °C in 50 mM citrate buffer, pH 4.8 with sodium azide 0.01% 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 mM potential cellobiose, six concentrations of Cel7A (4, 10, 42, 100, 417 and 1,000 μg mL^{-1}). Identical experiments with added initial cellobiose (7.5 and 15.0 mM) were also realized at the 5.0% and 2.5% (*w*/*v*) substrate concentrations and cellobiohydrolase Cel7A concentrations of 100 and 1,000 μg mL^{-1}.

For each assay, a complete progress curve was analyzed. In order to fit the experimental values with integrated Michaelis–Menten equation, substrate/enzyme ratios ([S] (μg mL^{-1})/[E] (μg mL^{-1})) smaller than 6.5 were not considered because they are in contradiction with usual assumptions of enzymatic kinetics since enzyme concentration should be much smaller than substrate concentration. The kinetic parameters from the integrated Michaelis–Menten equations were evaluated from non-linear least square regression using SAS software [8, 26, 32].

Results and Discussion

Theoretical Framework

Although competitive and non-competitive inhibition are the most readily accepted forms of Cel 7A inhibition by cellobiose [23] is advantageous to study other types of kinetics usually not considered. Thus, the mixed hyperbolic inhibition model with parabolic inhibition and also substrate inhibition MHIPSI (Fig. 1), assumed as hypothesis in this paper, is based on the assumption that cellulases may form productive (ES) and non-productive (ES′) enzyme-substrate complexes. While the formation of productive ES

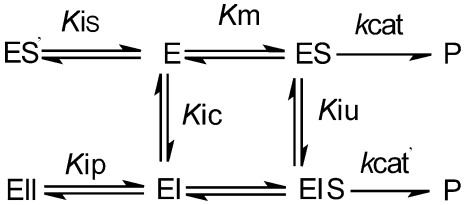

Fig. 1 Mixed hyperbolic (partial) inhibition model with substrate and parabolic inhibition ***MHIPSI*** where: *E* enzyme, *ES* enzyme substrate complex, *S*,*S*′ substrate (cellulose), *EIS* enzyme substrate inhibitor complex, *EI*, *EII* enzyme inhibitor complex, *P* product (cellobiose), K_m Michaelis–Menten constant, K_{ic}, K_{iu}, K_{ip}, K_{is} inhibition constants and k_{cat}, $k_{cat'}$ rate constants. Mixed hyperbolic inhibition model with substrate and parabolic inhibition MHI can be obtained by simplification of previous model assuming K_{is}, K_{ip}, as infinite. Thus, it is possible to obtain the following linear (total) inhibition models ($k_{cat'}$=0): Mixed inhibition model MLI (K_{ip} and K_{is}=∞); competitive inhibition CLI (K_{iu}, K_{ip}, and K_{is}=∞); non-competitive inhibition NCLI (K_{ic}=K_{iu}, K_{ip} and K_{is}=∞); uncompetitive inhibition UI (K_{ic}, K_{ip} and K_{is}=∞). A model without inhibition WI can be obtained by simplification of previous model assuming K_{is}, K_{ip}, K_{ic}, K_{iu}, as infinite

complexes leads to cellulose hydrolysis and the concomitant release of enzyme and cellobiose, non-productive ES′ complexes are catalytically inactive since the enzyme binds to the substrate but is not able to catalyze any reaction (formation of non-productive substrate enzyme complex). A possible biological interpretation of this hypothesis would suggest that the cellulases are segregated on the outside of the cell, and it is not energetically advantageous that the enzyme should move away from where it was produced. Thus, the synergism with other enzymes (e.g. other cellulases, ligninases and hemicellulases) can give rise to new ES complexes productive and justify the enzyme stay near the place where it was produced. This hypothesis also was considered in general terms by Klyosov and Rabinovich [33], Kim et al. [25], Nidetzky et al. [20] and Väljamäe et al. [34].

The reason for considering the parabolic inhibition was due to the knowledge that catalytic domains of Cel7A, and the exo-cellulases in general, have particular three-dimensional structures and a long tunnel-shaped active site with several binding sub-sites [35]. It is not surprising, therefore, that the model assumes a parabolic inhibition. Furthermore, if catalytic domains of Cel7A have several binding sub-sites, it is logical that the complex ESI can be productive. Thus, the hyperbolic (partial) inhibition was assumed as a hypothesis that must be investigated. Although hyperbolic inhibitions are not much studied in part because the difficulties of traditional methods, its study is of great interest [29].

Assuming the general model (MHIPSI) in Fig. 1 and the Michaelis–Menten Kinetic, we obtain the following rate equation:

$$v = \frac{V(1 + k_{\mathrm{cat}'}.P/(k_{\mathrm{cat}}.K_{\mathrm{iu}}))So}{K_{\mathrm{m}}\left(\left(1 + \frac{So}{K_{\mathrm{is}}}\right) + \frac{P}{K_{\mathrm{ic}}}\left(1 + \frac{P}{K_{\mathrm{ip}}}\right)\right) + So\left(1 + \frac{P}{K_{\mathrm{iu}}}\right)}$$

(with the symbols explained in the nomenclature and Fig. 1).

The equation can be integrated assuming that the substrate concentration decreases with time ($[So]-[St]+[Po]=[P]$).

$$-\frac{1}{dtV} = \left(\frac{St\left(1+\frac{k_{\mathrm{cat}'}}{k_{\mathrm{cat}}}\left(\frac{So}{K_{\mathrm{iu}}}-\frac{S}{K_{\mathrm{iu}}}+\frac{Po}{K_{\mathrm{iu}}}\right)\right)}{K_{\mathrm{m}}\left(1+\frac{St}{K_{\mathrm{is}}}+\left(\frac{So}{K_{\mathrm{ic}}}-\frac{St}{K_{\mathrm{ic}}}+\frac{Po}{K_{\mathrm{ic}}}\right)\left(1+\frac{So}{K_{\mathrm{ip}}}-\frac{St}{K_{\mathrm{ip}}}+\frac{Po}{K_{\mathrm{ip}}}\right)\right)+St\left(1+\frac{So}{K_{\mathrm{iu}}}-\frac{St}{K_{\mathrm{iu}}}+\frac{Po}{K_{\mathrm{iu}}}\right)}\right)dS$$

$$-dtV = \frac{\left(\frac{K_{\mathrm{m}}So^2}{K_{\mathrm{ic}}K_{\mathrm{ip}}}+K_{\mathrm{m}}+\frac{K_{\mathrm{m}}So}{K_{\mathrm{ic}}}+\frac{K_{\mathrm{m}}Po}{K_{\mathrm{ic}}}+\frac{K_{\mathrm{m}}Po^2}{K_{\mathrm{ic}}K_{\mathrm{ip}}}+\frac{2K_{\mathrm{m}}SoPo}{K_{\mathrm{ic}}K_{\mathrm{ip}}}\right)+\left(\frac{K_{\mathrm{m}}}{K_{\mathrm{is}}}-\frac{K_{\mathrm{m}}}{K_{\mathrm{ic}}}-\frac{K_{\mathrm{m}}2So}{K_{\mathrm{ic}}K_{\mathrm{ip}}}+1-\frac{K_{\mathrm{m}}2Po}{K_{\mathrm{ic}}K_{\mathrm{ip}}}+\frac{Po}{K_{\mathrm{iu}}}+\frac{So}{K_{\mathrm{iu}}}\right)S+\left(\frac{K_{\mathrm{m}}}{K_{\mathrm{ic}}K_{\mathrm{ip}}}-\frac{1}{K_{\mathrm{iu}}}\right)S^2}{(1+KvSo+KvPo)S+(-Kv)S^2}dS$$

Where $Kv=k_{\mathrm{cat}'}/(k_{\mathrm{cat}}K_{\mathrm{iu}})$

Assuming the following correspondence between the letters B–F, the equation can be represented:

$$-dtV = \frac{(B) + (C)S + (D)S^2}{(E)S + (F)S^2}dS$$

Where:

$$B = K_{\mathrm{m}}\left(\frac{So}{K_{\mathrm{ic}}} + \frac{Po}{K_{\mathrm{ic}}} + 1 + \frac{So^2}{K_{\mathrm{ic}}K_{\mathrm{ip}}} + \frac{2SoPo}{K_{\mathrm{ip}}K_{\mathrm{ic}}} + \frac{Po^2}{K_{\mathrm{ip}}K_{\mathrm{ic}}}\right)$$

$$C = \left(1 - \frac{K_{\mathrm{m}}}{K_{\mathrm{ic}}} + \frac{So}{K_{\mathrm{iu}}} + \frac{Po}{K_{\mathrm{iu}}} + \frac{K_{\mathrm{m}}}{K_{\mathrm{is}}} - \frac{2K_{\mathrm{m}}So}{K_{\mathrm{ic}}K_{\mathrm{ip}}} - \frac{2K_{\mathrm{m}}Po}{K_{\mathrm{ic}}K_{\mathrm{ip}}}\right)$$

$$D = \left(\frac{K_{\mathrm{m}}}{K_{\mathrm{ic}}K_{\mathrm{ip}}} - \frac{1}{K_{\mathrm{iu}}}\right) \quad E = \left(1 + \frac{k_{\mathrm{cat}'}}{k_{\mathrm{cat}}K_{\mathrm{iu}}}So + \frac{k_{\mathrm{cat}'}}{k_{\mathrm{cat}}K_{\mathrm{iu}}}Po\right) \quad F = \left(-\frac{k_{\mathrm{cat}'}}{k_{\mathrm{cat}}K_{\mathrm{iu}}}\right)$$

Table 2 Integrated Michaelis–Menten equations obtained from the models explained in Fig. 1

	Kinetic models	Integrated equations
WI	Without inhibition	$t = -\frac{1}{V}\left\{K_{\mathrm{m}} \ln \frac{St}{So} + (St - So)\right\}$
CLI	Competitive linear inhibition	$t = -\frac{1}{V}\left\{K_m\left(\frac{So}{K_{\mathrm{ic}}} + \frac{Po}{K_{\mathrm{ic}}} + 1\right) \ln \frac{St}{So} + \left(1 - \frac{K_{\mathrm{m}}}{K_{\mathrm{ic}}}\right)(St - So)\right\}$
NCLI	Non-competitive linear inhibition (K_{ic}=K_{iu})	$t = -\frac{1}{V}\left\{K_{\mathrm{m}}\left(\frac{So}{K_{\mathrm{iu}}} + \frac{Po}{K_{\mathrm{iu}}} + 1\right) \ln \frac{St}{So} + \left(1 - \frac{K_{\mathrm{m}}}{K_{\mathrm{iu}}} + \frac{Po}{K_{\mathrm{iu}}} + \frac{So}{K_{\mathrm{iu}}}\right)(St - So) - \frac{1}{2K_{\mathrm{iu}}}(St^2 - So^2)\right\}$
UCLI	Uncompetitive linear inhibition	$t = -\frac{1}{V}\left\{K_{\mathrm{m}} \ln \frac{St}{So} + \left(1 + \frac{So}{K_{\mathrm{iu}}} + \frac{Po}{K_{\mathrm{iu}}}\right)(St - So) + \left(-\frac{1}{2K_{\mathrm{iu}}}\right)(St^2 - So^2)\right\}$
MLI	Mixed linear inhibition	$t = -\frac{1}{V}\left\{K_{\mathrm{m}}\left(\frac{[So]}{K_{\mathrm{ic}}} + \frac{[Po]}{K_{\mathrm{ic}}} + 1\right) \ln \frac{[St]}{[So]} + \left(1 - \frac{K_{\mathrm{m}}}{K_{\mathrm{ic}}} + \frac{[So]}{K_{\mathrm{iu}}} + \frac{[Po]}{K_{\mathrm{iu}}}\right)([St] - [So]) + \left(-\frac{1}{2K_{\mathrm{iu}}}\right)([St^2] - [So^2])\right\}$
MHI	Mixed hyperbolic inhibition	$t = -\frac{1}{V}\left\{\left(\frac{B}{E} - \frac{C}{F} + \frac{DE}{F^2}\right) \ln\left(\frac{E + FSo}{E + FSt}\right) + \frac{B}{E} \ln \frac{St}{So} + \frac{D}{F}(St - So)\right\}$ $B = K_{\mathrm{m}}\left(\frac{So}{K_{\mathrm{ic}}} + \frac{Po}{K_{\mathrm{ic}}} + 1\right) \quad C = \left(1 - \frac{K_{\mathrm{m}}}{K_{\mathrm{ic}}} + \frac{So}{K_{\mathrm{iu}}} + \frac{Po}{K_{\mathrm{iu}}}\right) \quad D = \left(-\frac{1}{K_{\mathrm{iu}}}\right)$ $E = \left(1 + \frac{k_{\mathrm{cat}'}}{k_{\mathrm{cat}}K_{\mathrm{iu}}} So + \frac{k_{\mathrm{cat}'}}{k_{\mathrm{cat}}K_{\mathrm{iu}}} Po\right) F = \left(-\frac{k_{\mathrm{ca}t'}}{k_{\mathrm{cat}}K_{\mathrm{iu}}}\right)$
MHIPSI	Mixed hyperbolic inhibition with parabolic and substrate inhibition	$t = -\frac{1}{V}\left\{\left(\frac{B}{E} - \frac{C}{F} + \frac{DE}{F^2}\right) \ln\left(\frac{E + FSo}{E + FSt}\right) + \frac{B}{E} \ln \frac{St}{So} + \frac{D}{F}(St - So)\right\}$ $B = K_{\mathrm{m}}\left(\frac{So}{K_{\mathrm{ie}}} + \frac{Po}{K_{\mathrm{ic}}} + 1 + \frac{So^2}{K_{\mathrm{ic}}K_{\mathrm{ip}}} + \frac{2SoPo}{K_{\mathrm{ip}}K_{\mathrm{ic}}} + \frac{Po^2}{K_{\mathrm{ip}}K_{\mathrm{ic}}}\right)$ $C = \left(1 - \frac{K_{\mathrm{m}}}{K_{\mathrm{ic}}} + \frac{So}{K_{\mathrm{iu}}} + \frac{Po}{K_{\mathrm{iu}}} + \frac{K_{\mathrm{m}}}{K_{\mathrm{is}}} - \frac{2K_{\mathrm{m}}So}{K_{\mathrm{ic}}K_{\mathrm{ip}}} - \frac{2K_{\mathrm{m}}Po}{K_{\mathrm{ic}}K_{\mathrm{ip}}}\right)$ $D = \left(\frac{K_{\mathrm{m}}}{K_{\mathrm{ic}}K_{\mathrm{ip}}} - \frac{1}{K_{\mathrm{iu}}}\right) \quad E = \left(1 + \frac{k_{\mathrm{cat}'}}{k_{\mathrm{cat}}K_{\mathrm{iu}}} So + \frac{k_{\mathrm{cat}'}}{k_{\mathrm{cat}}K_{\mathrm{iu}}} Po\right) F = \left(-\frac{k_{\mathrm{cat}'}}{k_{\mathrm{cat}}K_{\mathrm{iu}}}\right)$

Table 3 Summary of statistical parameters for different models in assays with Cel7A

	WI	CLI	NCLI	UCLI	MLI	MHI	MHIPSI
SSE	161.423	58.100	83.742	80.99	57.943	52.533	52.211
p	2	3	3	3	4	5	7
n	563	563	563	563	563	563	563

With initial presence of cellobiose; the models are explained in Fig. 1; see also the text

Then

$$\int_{o}^{t} dtV = \int_{So}^{St} \frac{B}{ES + FS^2} dS + \int_{So}^{St} \frac{C}{E + FS} dS + \int_{So}^{St} \frac{DS}{E + FS} dS$$

To obtain the following equation:

$$t = -\frac{1}{V}\left(\frac{B}{E}\ln\frac{St}{So} + \left(\frac{C}{F} - \frac{DE}{F^2} - \frac{B}{E}\right)\ln\frac{E + FSt}{E + FSo} + \frac{D}{F}(St - So)\right)$$

This equation can be simplified giving rise to model with fewer constants but always hyperbolic. In Table 2, the hyperbolic mixed inhibition model (MHI) is presented and can be obtained by simplification of MHIPSI. The process of integration assumed k_{cat}, different from zero; thus, linear models cannot be obtained by simplification of MHI, which implies that linear and hyperbolic models are not nested. Linear models previously published [26] are also included in Table 2.

Discrimination and Obtained Constants

The kinetic investigation consists of two parts: discrimination between available models and parameter estimation. Furthermore, with Michaelis–Menten integrated equation, it is possible to study the product inhibition without initial addition of inhibitor. Thus, two types of analysis were performed: model discrimination with data from assays obtained in the presence of cellobiose initially added (Tables 3, 4 and 5) and another discrimination without initial addition of cellobiose (Tables 6, 7 and 8).

Table 4 Summary of comparing models using AIC in assays with the Cel7A with initial presence of cellobiose

Models A/B	SSE_A	SSE_B	Number	$k_A\ p_A+1$	$k_B\ p_B+1$	AIC_{cA}	AIC_{cB}	ΔAIC_c	Probability B correct (%)
WI/CLI	161.423	58.100	563	3	4	−697.3	−1270.6	−573.3	100
CLI/MLI	58.100	57.943	563	4	5	−1270.6	−1270.1	0.5	44
CLI/MHI	58.100	52.533	563	4	6	−1270.6	−1323.2	−52.6	100
CLI/MHIPSI	58.100	52.211	563	4	8	−1270.6	−1322.8	−52.0	100
MHI/MHIPSI	52.533	52.211	563	6	8	−1323.4	−1322.8	0.65	42

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

Table 5 Summary of obtained constants for the MHI model in assays with Cel 7A

K_m (mM)	K_{ic} (mM)	K_{iu} (mM)	K_{cat} (h^{-1})	$K_{cat'}$ (h^{-1})
5.0±1.2	0.029±0.007	1.1±0.4	3.6±0.5	0.2±0.03

With initial presence of cellobiose

To discriminate between models with the same number of parameters (CLI, NCLI and UCLI), it only is necessary to consider SSE value. Thus, the CLI model is preferable than UCLI and NCLI because the SSE value is lower (Table 3). Therefore, it is only necessary to compare the pairs with different number of parameters WI/CLI, CLI/MLI, CLI/MHI, CLI/MHIPSI and MHI/MHIPSI. The inspection of Table 4 (with initial presence of cellobiose) results that MHI model is preferable as explained bellow.

Incorporating multiple adjustable parameters into a model increases the possibility of fitting experimental data with an incorrect model. Nevertheless, there are several methodologies that permit to compare and discriminate models with different number of parameters such as extra sum-of-square F test (e.g. Mannervik [36] discrimination) or Akaike information criterion (AIC) [37, 38]. The theoretical basis of Akaike's method combines maximum likelihood theory, information theory and the concept of entropy of information. Both methodologies are used to discriminate models with different number of parameters and the same number of experimental points. The disadvantage of Mannervik methodology is that the F ratio test is not applicable to non-nested models, and significance levels (99%, 95%, 90%, ...) are arbitrary. To discriminate models, the use of AIC has the advantages that the models assumed can be nested or non-nested and the results give a continuous scale for model plausibility [38–40]. As explained previously, it is important to point out that during the integration, it was assumed that $k_{cat'}$ is different from zero and so the linear models cannot be obtained by simplification of MHI. Thus, it is not possible to use the Mannervik discrimination [36] since the models are not nested.

The AIC is defined by the following equation [37, 38]:

$$\mathrm{AIC} = n\ln(\mathrm{SSE}/n) + 2k$$

Where n is the number of data points, k is the number of parameters fit by the regression plus one (k=p+1) and SSE is the sum of the square of vertical distances of points from the curve.

The correct AIC (AIC_C) value has an advantage when compared with AIC (if sample data are large, there are only a few corrections because the denominator is much greater than the numerator):

$$\mathrm{AIC_C} = \mathrm{AIC} + (2k(k+1)/(n-k-1))$$

Table 6 Summary of statistical parameters for different models in assays with Cel7A

	WI	CLI	NCLI	UCLI	MLI	MHI	MHIPSI
SSE	120.315	47.837	62.901	80.363	47.233	46.078	46.078
p	2	3	3	3	4	5	7
n	467	467	467	467	467	467	467

Without initial presence of cellobiose; the models are explained in Fig. 1; see also the text

Table 7 Summary of comparing models using AIC in assays with the Cel7A without initial presence of cellobiose

Models A/B	SSE_A	SSE_B	Number	$k_A\ p_A+1$	$k_B\ p_B+1$	AIC_{cA}	AIC_{cB}	ΔAIC_c	Probability B correct (%)
WI/CLI	120.315	47.837	467	3	4	2598.6	2169.9	−428.7	100
CLI/MLI	47.837	47.233	467	4	5	2169.9	2166.0	−3.9	87
CLI/MHI	47.837	46.078	467	4	6	2169.9	2156.5	−13.4	100
MLI/MHI	47.233	46.078	467	5	6	2166.0	2156.5	−9.5	99

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

The model with lower AIC_C value is the model more likely to be correct. The following equation gives the probability that model B is correct:

$$\text{Probability} = \left(e^{-0.5\Delta}/\left(1+e^{-0.5\Delta}\right)\right)$$

Where Δ is the difference between AIC_c values ($AIC_c = AIC_{cb} - AIC_{ca}$) where A model is the simpler one.

By Table 4 inspection, it is evident that MHI model is the most appropriate (results with initial presence of cellobiose) and the likelihood of this happening is 58% when compared with the best second model (MHIPSI) which has only a probability of 42%. It is important to note that the results of the models give sum of squares error (SSE_{MHIPSI} and SSE_{MHI}) that differ only by a small amount. As the MHI is the best model, it is now possible to summarize the constants obtained with this model (Table 5). Then, in studies with initial presence of cellobiose, MHI model is better than any other model. Nevertheless, the inhibition constant K_{ic} (when compared with other inhibition constants, Table 5) shows that competitive inhibition have a great importance in the process ($K_{ic} \ll K_{iu}$), though assuming the possibility of ESI to be a productive complex, the inhibition selected becomes mixed hyperbolic. Otherwise, $k_{cat'}$ constant is 10 times lower than k_{cat}, but the kinetic is clearly hyperbolic. The constants (K_m, K_{ic} and k_{cat}) are in the same order as the magnitude previously discussed and determined [8, 26, 32].

AIC data analyses obtained without initial presence of cellobiose also discriminate the MHI model (Tables 6 and 7), and the constants obtained are presented in Table 8. An example of time course hydrolysis and fitted curves obtained with MHI model is presented in Fig. 2. It is important to point out that the initial product of reaction obtained with the enzyme Cel7A is the β-anomer, and the commercial cellobiose added is a racemic mixture between two anomers (α and β). The principal advantage to determine inhibition constants of reaction product (cellobiose) without addition of the inhibitor is the certitude that the inhibition is due to the correct cellobiose anomer resulting from the reaction of hydrolysis. Nevertheless, this assumption had no influence on the kinetic model obtained. Analyzing

Table 8 Summary of obtained constants for the MHI model in assays with Cel 7A

K_m (mM)	K_{ic} (mM)	K_{iu} (mM)	K_{1cat} (h^{-1})	K_{2cat} (h^{-1})
4.7±1.2	0.029±0.007	1.5±0.6	3.2±0.5	0.2±0.05

Without initial presence of cellobiose

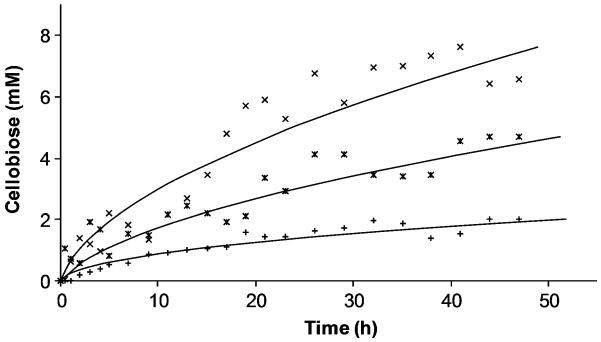

Fig. 2 Experimental points and fitted curves of cellulose hydrolysis: *plus sign* 0.25% (*w*/*v*) Avicel and 100 μg mL^{-1} enzyme; *asterisk* 5% (*w*/*v*) Avicel and 42 μg mL^{-1} enzyme; *multiplication symbol* 5% (*w*/*v*) Avicel and 100 μg mL^{-1} enzyme. Kinetic model accounting MHI was used to fit curves using estimated constants (Table 8)

Tables 6, 7 and 8, we can conclude that it is not necessary to utilize the AIC methodology to discriminate between hyperbolic models once MHIPSI and MHI SSE values are equal. Thus, MHI model better explains the results because it has fewer parameters.

It is important to refer that linear model with parabolic and substrate inhibition was previously studied and also pointed out that these inhibitions are not the principal constraint to cellulose hydrolysis [8]. These conclusions are supported by results from Tables 4 and 7, though, in the present paper, linear and hyperbolic models were compared, and furthermore, the substrate concentration was considered as varying during the reaction time ($[So] - [St] = [P]$) differently than was assumed previously [8]. Nevertheless, it is important to point out the assumption that the complex ESI is productive: change the best model previously discriminated (CI model) to a model that now incorporates mixed hyperbolic inhibition (MHI model) as the model that better explains the results obtained. As previously explained, the assumption that the complex ESI is productive is a logical hypothesis since catalytic domains of Cel7A have several binding sub-sites [35].

The possibility of utilizing this methodology to determine kinetic constants, in general kinetic studies, and the possibility of developing spectrophotometer software to indicate "on line" the type of inhibition and kinetic constants seem to us important to point out. In fact, integrated equations allow us to determine accurate kinetic parameters even when the reaction product is an inhibitor [2].

Conclusions

It was found that cellulose hydrolysis by Cel7A is inhibited by cellobiose (final reaction product) and follows a MHI model. Other models that incorporate improvements concerning inhibition by substrate and also parabolic inhibition only increase the modulation performances very slightly. However, these models should not be preferred over the MHI model because the goodness-of-fit increase was not supported by model discrimination according to Akaike information criterion. The results point out that non-productive enzyme-substrate complexes and parabolic inhibition are not the principal constraints in enzymatic cellulose hydrolysis. Since both assays with and without initial cellobiose give similar results, this reinforced the assumption that MHI model accurately described the time course kinetics of cellulose hydrolysis.

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