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Published in:
Analytical Biochemistry

DOI:
10.1016/j.ab.2019.113411

Publication date:
2019

Document Version
Peer reviewed version

Citation for published version (APA):
Kari, J., Christensen, S. J., Wienecke Andersen, M., Sellés Baiget, S., Borch, K., & Westh, P. (2019). A practical approach to steady-state kinetic analysis of cellulases acting on their natural insoluble substrate. *Analytical Biochemistry*, *586*(586), Article 113411. https://doi.org/10.1016/j.ab.2019.113411

Analytical Biochemistry xxx (xxxx) xxx-xxx

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: http://ees.elsevier.com

A practical approach to steady-state kinetic analysis of cellulases acting on their natural insoluble substrate

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ARTICLE INFO

Keywords
Heterogeneous enzyme catalysis
Steady state kinetics
Initial rate measurements
Inverse Michaelis Menten equation
Interfacial enzyme reaction
Cellobiohydrolase

ABSTRACT

Measurement of steady-state rates (v_{SS}) is straightforward in standard enzymology with soluble substrate, and it has been instrumental for comparative biochemical analyses within this area. For insoluble substrate, however, experimental values of v_{ss} remain controversial, and this has strongly limited the amount and quality of comparative analyses for cellulases and other enzymes that act on the surface of an insoluble substrate. In the current work, we have measured progress curves over a wide range of conditions for two cellulases, TrCel6A and TrCel7A from *Trichoderma reesei*, acting on their natural, insoluble substrate, cellulose. Based on this, we consider practical compromises for the determination of experimental v_{SS} values, and propose a basic protocol that provides representative reaction rates and is experimentally simple so that larger groups of enzymes and conditions can be readily assayed with standard laboratory equipment. We surmise that the suggested experimental approach can be useful in comparative biochemical studies of cellulases; an area that remains poorly developed.

1. Introduction

Cellulose is the most abundant organic molecule in the Biosphere, and a wide range of microorganisms has evolved the ability to use it as a source of carbon and energy. These microorganisms secrete an arsenal of catabolic enzymes, which have attracted considerable interest within both applied- and fundamental enzymology. Applied aspects of cellulases center on the enzymatic conversion of lignocellulosic biomass to fermentable sugars (so-called saccharification), which is a key step in upcoming biorefineries [1,2]. From a fundamental point of view, cellulases are intriguing because they are evolved to attack a polymeric, crystalline substrate with very poor accessibility of the scissile β-1,4 glucosidic bond. Adaptation to these conditions have promoted distinctive enzyme structures including the non-covalent association into multi-enzyme complexes called cellulosomes [3], and the multi-domain structure of fungal cellulases [4]. These diverse structures of cellulolytic enzymes often entail complex modes of substrate interactions that include both ligand binding in extensive active sites and other attractive forces between the enzyme and the surface of the insoluble substrate [5]. This network of interactions has only been investigated for a small subset of cellulases, and its roles for enzymatic efficacy remains elusive even within this subset. Recently, progress within structural- [6–8], computational- [9,10], and imaging [11,12] studies have led to an emerging understanding of elementary steps that underlie the multifaceted enzyme process. Critical assessment of such elementary steps and ultimately the formulation of realistic micro-kinetic reaction schemes will require systematic, comparative investigations of cellulase kinetics. However, this latter area remains underdeveloped and functional analyses of cellulases usually rely on empirical comparisons of progress curves or end-point measurements. Parameters derived from this type of experiments are often ambiguous with respect to physical meaning and unlikely to be valid outside the experimental conditions under which they are measured. More systematic and theoretically sound approaches to cellulase kinetics have been challenged by several general factors including a shortage of quantitative assays [13,14] and straightforward models for the interpretation of kinetic data [15,16]. The biggest single hurdle, however, has probably been the ubiquitous slowdown observed for cellulolytic processes [17–21]. This manifests itself as a continuously falling rate even when factors such as inhibition, inactivation and substrate depletion appear negligible. Molecular origins of the slowdown have been discussed intensively and proposed to rely on factors such as substrate crystallinity, substrate reactivity, substrate accessibility and fractal kinetics (see Refs. [17,22] for reviews).

From the point of view of kinetic modeling, the slowdown and concomitant lack of a linear part in the progress curve means that it is

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https://doi.org/10.1016/j.ab.2019.113411
Received 10 July 2019; Received in revised form 26 August 2019; Accepted 28 August 2019
Available online xxx
0003-2697/

difficult to assess when and whether the quasi-steady state (QSS) assumption can be applied. At the current stage, it appears that an unequivocal identification of a QSS regime for cellulolytic enzyme reactions cannot be readily made and perhaps, for this reason, progress curves remain the preferred means of conveying functional data. Progress curves obviously provides important knowledge, but as stated in the authoritative textbook by Cornish-Bowden [23], one essential learning from the work of Michaelis and Menten was that *analysis in terms of initial rates was much simpler than struggling with time courses*, and this point has been amply confirmed for enzyme reactions in the bulk. The advantages of switching from time-course to initial rates might be even greater for cellulases and other enzymes that act on the interface of an insoluble substrate. This is because progress curves for these reactions are influenced by a gradual modification of the substrate surface [17]. Although attempts have been made to quantify the morphological changes in cellulose during enzymatic breakdown [24–27], such effects are at present poorly understood on a molecular level [16], and hence difficult to account for in mathematical descriptions of the progress curve. In light of this, it appears worthwhile to seek principles and protocols for the estimation of representative steady-state rates for cellulases, even if this entails assumptions and compromises that are coarser than in conventional enzyme kinetics.

Here, we report progress curves for the two cellobiohydrolases TrCel6A and TrCel7A from the filamentous fungus *Trichoderma reesei* acting on microcrystalline cellulose. The experiments were designed to cover a wide range of enzyme concentrations and substrate loads in order to saturate both the substrate with enzyme and the enzyme with substrate. Our goal is to use this comprehensive (labor intensive) data set to identify faster protocols that can be used as the experimental foundation for comparative biochemical analyses of cellulases based on simple steady state theory.

2. Materials and methods

All experiments were conducted in a standard 50 mM acetate buffer, pH 5.0.

Enzymes. The two cellobiohydrolases from *Trichoderma reesei*, TrCel6A and TrCel7A were heterologously expressed in *Aspergillus oryzae* and purified as described previously [28,29].

Progress curves. Both enzymes were investigated in two experimental series, which were set up to attain respectively saturation of enzyme with substrate and saturation of substrate with enzyme. For the enzyme saturation experiments we used a low, constant enzyme concentration (0.1 μM for TrCel7A and 0.025 μM for TrCel6A), while the substrate load was varied up to 100 g/L (see actual loads in Fig. 1A and C). In substrate saturation experiments, we applied a low, constant substrate load (5 g/L), while the enzyme concentration was varied up to 10 μM (see concentrations Fig. 1B and D). All reactions were carried out in a 15 ml thermostated beaker at 25 °C with magnetic stirring (500 RPM). We used microcrystalline cellulose from wood (Avicel PH101, Sigma-Aldrich, St. Louis, MO) as substrate, and injected enzyme at t = 0 to start the reaction. At selected time-points (specified by symbols in Fig. 1), we retrieved subsets of 500 μl from the beaker and immediately quenched the reaction by mixing into a vial with an equal amount of 0.1 M NaOH. The reaction was monitored for a total of 1 h and we retrieved a total of 20–25 samples for each progress curve. Cellulose was separated from the quenched samples by centrifugation (1500 RCF, 5 min) and the supernatants were analyzed for their content of glucose, cellobiose, and cellotriose by High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD). An 8 point standard curve for both glucose, cellobiose, and cellotriose was used as external standard. All measurements were repeated in duplicates.

3. Results

Fig. 1 shows progress curves for experiments with respectively enzyme saturation (panels A and C) and substrate saturation (panel B and D). It appears that the hydrolysis rate decreases rapidly over the first 5–10 min for both TrCel7A and TrCel6A. For TrCel7A the initial slowdown was particular pronounced in the enzyme saturation experiments (Fig. 1A), while the substrate saturation experiments (Fig. 1B) showed a minor slowdown over the first 5–10 min. For TrCel6A we observed the opposite behavior. This enzyme showed a minor initial slowdown in the enzyme saturation experiments (Fig. 1C) while the initial change in rate was more pronounced in the substrate saturation experiments (Fig. 1D). In all experiments the initial, fast decay in activity during the first 10 min was replaced by a much slower decay (i.e. near linear progress curves) in the time domain from 10 to 60 min.

4. Discussion

Kinetic characterization makes up an essential part in studies of any enzyme reaction. For cellulolytic enzymes, however, this area remains poorly developed and this hampers for example the implementation of comparative biochemical analyses. One reason for the limited occurrence of rigorous kinetic work is controversies regarding the specification of a steady-state reaction rate, v_{SS}, on the basis of non-linear progress curves. Obviously, non-linearity calls for caution in the application of steady-state approaches, but it does not necessarily prohibit their use. Some aspects of this has been discussed elsewhere [30], and it was concluded that the steady-state assumption could be valid for cellulases provided that the substrate was in large excess. However, excess implies a large stoichiometric (*i.e.* molar) surplus of the substrate, and this is not straightforward to define and assess for an insoluble compound like cellulose. One way to approach this is to require that the number of accessible, scissile bonds on the surface (sometimes called "attack-sites" or "productive binding sites"), far outnumbers enzyme molecules in the investigated system. This criteria for the heterogeneous system is analogous to the usual requirement of substrate excess for enzyme reactions in the bulk (see Ref. [31] for at detailed discussion). In addition to this conventional approach to steady state kinetics, it has been proposed that in the special case of insoluble substrate, the steady-state assumption can also be used under the opposite conditions, where the enzyme is in large excess [32–35]. If indeed the steady-state assumption can be applied to interfacial enzyme reactions with excess of either substrate or enzyme, it is worthwhile to seek convenient approaches to experimental values of v_{SS}. This could pave the way for comparative biochemical analyses of cellulases, and hence, in turn, a better understanding of these intriguing and industrial important enzymes. In the current work we address this through the analysis of a comprehensive set of progress curves for two cellulases. The overall goal is to use this more thorough data set to identify simplifications and compromises that allow reasonable estimates of v_{SS} by simple procedures.

Initially, we analyze the course of the progress curves with emphasis on what causes the observed nonlinearity. We will exclude product inhibition and physical instability of the enzymes from this discussion as earlier results regarding inhibition [36–39] and stability during hydrolysis [40] have shown that these factors have little or no effect on the slowdown under the current experimental conditions. The progress curves in Fig. 1 can roughly be divided into two time domains with respect to the slowdown. During the first 10 min, there is a significant deceleration in product formation, while the decline in rate is much less prominent in the later part (10–60 min) of the measured reaction (see Fig. 1 and Supplementary Material). The slowdown of TrCel7A and TrCel6A during the initial part of the reaction has previously been described by so-called burst kinetics [41–45], which entails a transient phase with a rate that exceeds v_{ss} [46]. The molecular origin of the burst-kinetics is probably slow release of unproductive, bound

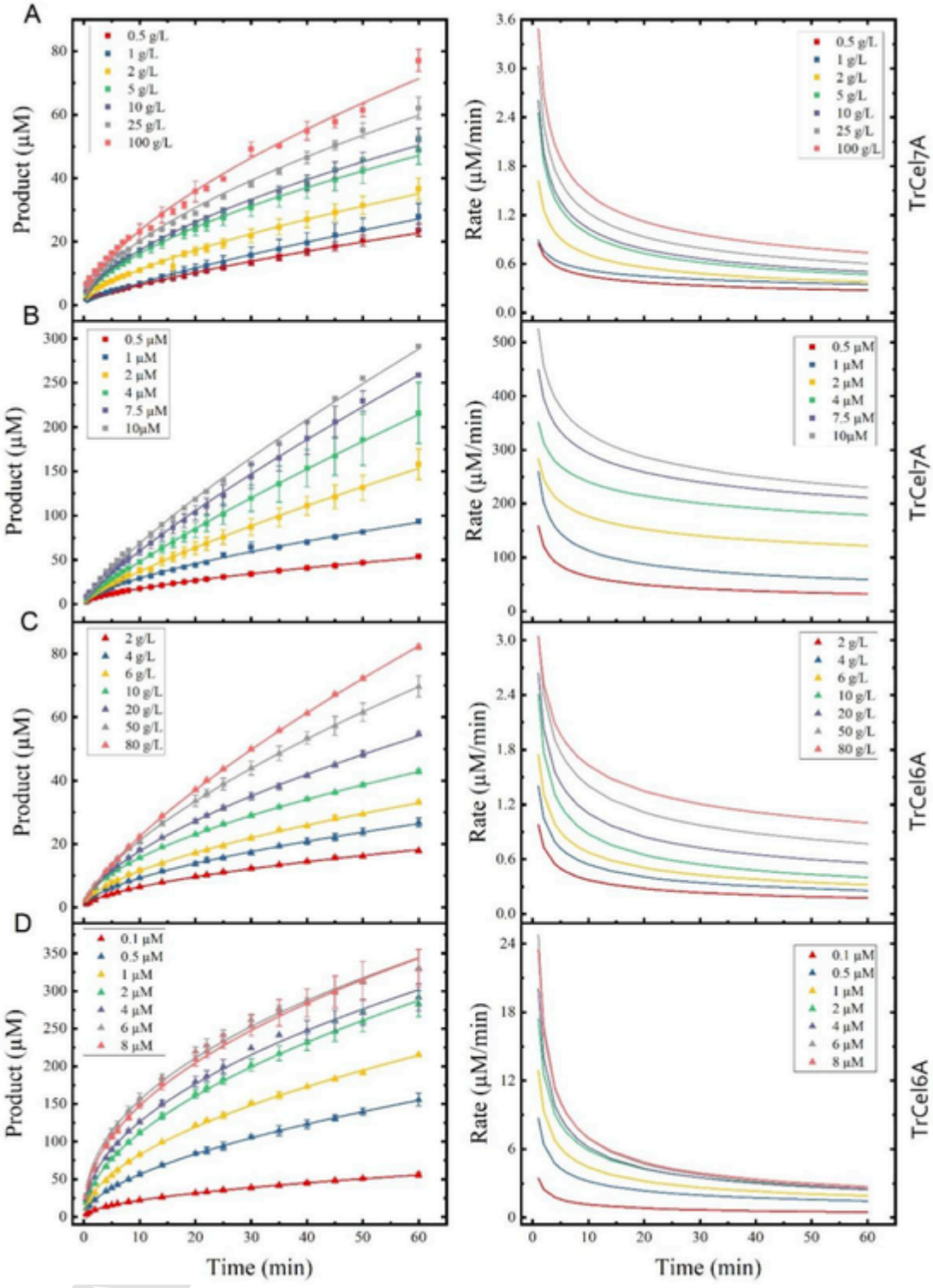

Fig. 1. Progress curves and derived rates for TrCel7A (*A, B*) and TrCel6A (*C, D*) acting on pure cellulose (Avicel) at 25 °C. *Left panels* show progress curves while *right panels* show the derivative of the fitted curves in the left panel and hence represents the reaction rate. Panels *A* and *C* show progress curves and rates for the enzyme saturation experiments. In these experiments, the enzyme concentration was fixed (0.1 μM TrCel7A, 0.025 μM TrCel6A) and the substrate load was varied. *B* and *D* show progress curves and rates for the substrate saturation experiments. In these experiments, the substrate load was fixed (5 g/L) and the enzyme concentration was varied as indicated in the figures. Product concentrations on the ordinates refers to the sum of glucose, cellobiose, and cellotriose. Solid lines represents best fit of a simple power function, $P(t) = At^B$. This serves to guide the eye in the progress curves and was also used to calculate reaction rates, $dP/dt = A(B\text{-}1)t^{(B-1)}$. For the substrate saturation experiments with TrCel7A (*B*), the progress curve with the highest enzyme concentration (10 μM) was only made once to save material.

enzymes [41,45–47] that build up in front of obstacles and irregularities on the cellulose surface and hence limit the processive movement of the cellobiohydrolases. High-speed AFM data for TrCel7A and TrCel6A has supported this idea by visualizing how these enzymes accumulate at the cellulose surface [12]. With respect to the results in Fig. 1, we suggest that the high initial rates are dominated by the burst phase. This

is inherently a pre-steady state regime [46], and it is hence inappropriate for determination of v_{SS}. It follows that the customary procedure of using the slope of the progress curve for t → 0 as a measure of the initial, steady state rate would overestimate the quasi-steady state rate (v_{SS}), and we advise against this strategy for cellulases.

In the second time domain (10–60 min) changes in the reaction rates are much less pronounced (Fig. 1). The weak slowdown observed in this regime is a hallmark of enzymatic conversion of cellulose and inherently related to the heterogeneous environment that the enzymes work in . [48]. Its molecular origin(s) has been the subject of intense debate as reflected in different reviews [16,17,22,49–51], and common interpretations include modifications in reactivity, crystallinity or accessibility of the substrate [18–21,48,52–56]. It is beyond the current scope to discuss these mechanisms, and we simply deduce that the rate falls slowly in this regime due to changes in substrate properties. It appears that quasi steady state is feasible over a limited time interval in this regime, and if indeed so, the next step is to find a practicable and consistent way to derive v_{ss} from the progress curve. The remainder of the discussion will focus on this task. The most straightforward approach to this is to determine the slope of the tangent at a fixed time (*c.f.* orange lines in Fig. 2). This "tangent method" is theoretically sound and hence appealing, but it is quite demanding from an experimental point of view because it requires several independent data points and possibly also the application of a fitting function to smoothen experimental scatter (as indeed used for the conversion between progress curve and rate in Fig. 1). Alternatively, one may use the slope of a secant as illustrated by green lines in Fig. 2. This latter approach is more workable because it only requires one data point and no fitting function (the rate is simply [P]/Δt). The main drawback of the secant method is that it systematically overestimates v_{SS} since (unlike the tangent) it incorporates the contribution from the preceding, faster part of the progress curve (particularly the transient burst). To compare the two different methods systematically, we first estimated reaction rates by the secant method using the measured product concentrations at all experimental time points of the progress-curves in Fig. 1. To estimate rates by the tangent method, we fitted a power law function, $P(t) = At^B$ to the progress curves as illustrated by the lines in the left column of Fig. 1. As seen for other cellulolytic reactions [57], this function generally fits the progress curves well. Next, we used

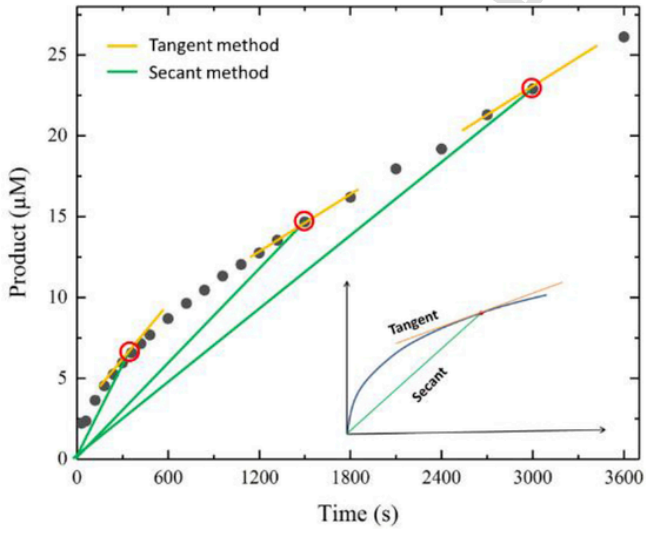

Fig. 2. Illustration of the secant- and tangent methods to derive rates from a progress curve. Black symbols represent the experimental values in a progress curve (taken from Fig. 1). The methods are exemplified by three time-points (t = 300 s, t = 1400 s and t = 3000 s), which are marked with red circles on the progress curve. The secants (green) and tangents (orange) at these points are shown on the figure. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the differential coefficient, $dP/dt = A(B\text{-}1)t^{(B-1)}$ to compute the slope of the tangent. Once the parameters A and B have been found by fitting the power law equation to the progress curves, dP/dt can obviously be readily computed at any time-point. However, for simplicity we will use the average tangent slope of the near linear part of the progress curve (10–60 min) in the following.

The rates derived by either secant or tangent slopes were used in two separate steady state kinetic analyses. The first steady-state analysis followed many earlier examples, and used the conventional Michaelis Menten equation to rationalize data from experiments with an excess of substrate [30,58–62]. In practice, this implies a series of measurements with a constant, low enzyme concentration (E_0), and a number of different loads of Avicel (S_0). Results were analyzed with respect to equation [1], in which saturation reflects binding of essentially all enzyme to the substrate (*i.e.* saturation of enzyme with substrate)

$$v_{ss} = \frac{^{conv}V_{\max}S_0}{^{conv}K_m + S_0} \quad (1)$$

Earlier work has suggested that interfacial enzyme processes may also be described by an inverse (or reverse) approach, where the *enzyme* is in excess [30,32,34,35,63]. In this case, the experiments use a constant, low substrate Avicel load (S_0) and different enzyme concentrations (E_0). This type of measurements can be described by the so-called inverse Michaelis Menten equation, Eq (2). In this case, saturation implies that all "attack sites" on the substrate surface are complexed with an enzyme molecule (*i.e.* saturation of substrate with enzyme).

$$v_{ss} = \frac{^{inv}V_{\max}E_0}{^{inv}K_m + E_0} \quad (2)$$

The parameters $^{conv}V_{max}$ and $^{inv}V_{max}$ define the reaction rate at respectively enzyme- and substrate saturation. Similarly, $^{conv}K_M$ is the load of substrate at half-saturation and $^{inv}K_M$ is the concentration of enzyme at half-saturation. The kinetic analysis used here is rooted in simple Michaelis-Menten kinetics. Other approaches such as FBU measurements, fractal kinetics and stochastic methods [64–70] have been applied to characterize cellulases. Although these strategies are without questioning important for both comparative analysis and molecular insight into cellulolytic enzymes, the current work will solely address the application of steady-state kinetics.

We measured progress curves for TrCel6A and TrCel7A under both substrate excess (Fig. 1A and C) and enzyme excess (Fig. 1B and D), and estimated the associated rates at all experimental time points by the secant method as explained above. Rates from this secant-based analysis were used to make either conventional- or inverse Michaelis Menten plots (all 86 conventional- or inverse Michaelis Menten plots may be found in the Supplementary Information). We fitted eq [1]. to data made under substrate excess (conventional Michaelis Menten analysis) and eq [2]. to data made with enzyme excess (inverse Michaelis Menten analysis), and plotted the parameters ($^{conv}V_{max}$, $^{inv}V_{max}$, $^{conv}K_M$, and $^{inv}K_M$) as a function of time in Fig. 3. Fig. 3 also shows the kinetic parameters derived by the tangent method. These tangent-based parameters were in good agreement with earlier measurements performed either under similar conditions [71,72] or in a somewhat different experimental setup [45]. For instance, Jalak and Valjamae [45] reported an overall rate constant for TrCel7A acting on Avicel at 25 °C of 2–15 min^{-1} (0.033–0.25 s^{-1}) which is similar to what we find here $^{conv}V_{max}/E_0 = 0.15\ s^{-1}$ for TrCel7A. The most important result in Fig. 3 is the secant-based and tangent-based parameters tend to merge after 60 min. Specifically, three of four parameters ($^{conv}V_{max}$, $^{inv}V_{max}$ and $^{conv}K_M$) for TrCel7A were identical (within the standard deviation) for the two methods at t = 60min, while the last ($^{inv}K_M$) was slightly underestimated by the secant method. For TrCel6A, the deviation between the two methods was somewhat larger, but

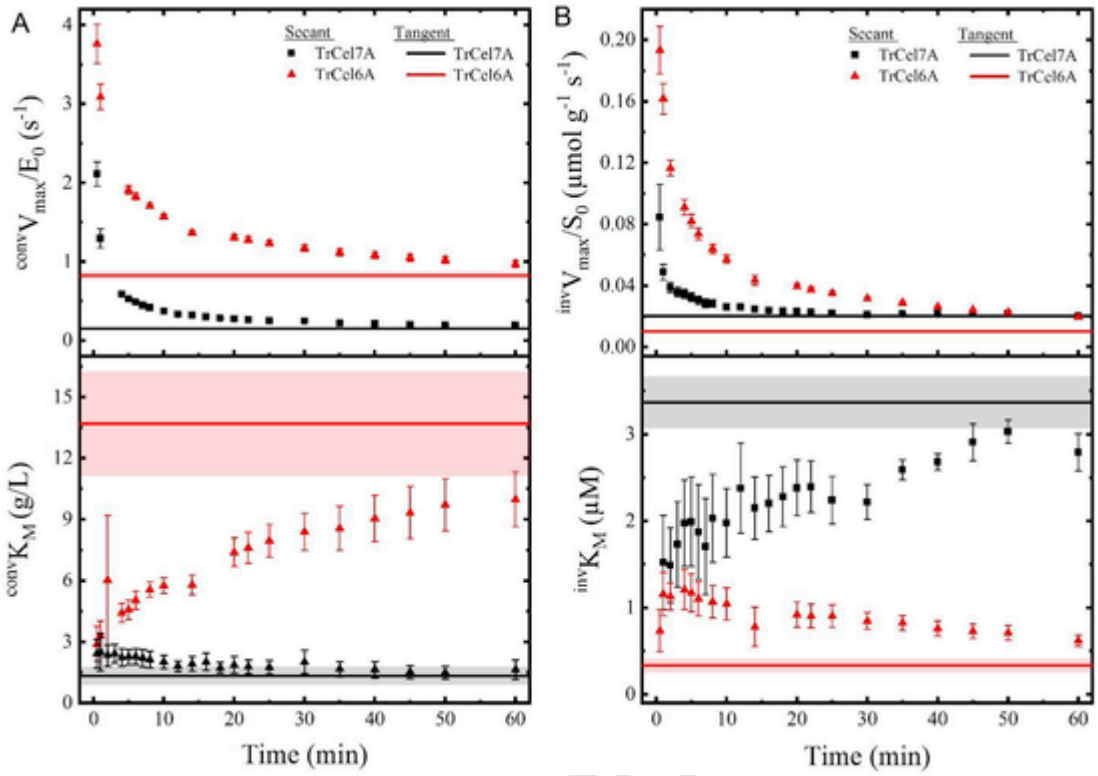

Fig. 3. Kinetic parameters derived using either the secant or tangent to determine quasi steady-state rates. Panel **A** shows the conventional Michaelis-Menten parameters (see eq. (1)) while panel **B** shows the Inverse Michaelis-Menten parameters (see eq. (2)). The kinetic parameters were derived from rates obtained at different time points as illustrated in Fig. S1 in the Supplementary Material, and plotted as a function of incubation time to illustrate how the secant methods asymptotically approach the parameters obtained with the tangent method. For simplicity only the average tangent slope of the near linear part of the progress curve (10–60 min) is shown. Shades around the lines representing data from the tangent method and error bars on the symbols for the secant method illustrate the standard error (±SE) of the non-linear regression analysis in Fig. S1. In some cases this error is too small to be clearly visible in the figure.

secant method at t = 60 min still provided parameters in reasonable agreement with the more fundamentally correct tangent method. We suggest that this result is of direct practical importance as it shows that a simple end-point measurement after 1 h can provide an acceptable estimate of the steady-state rate. In other words, the secant method for 1 h measurements appears to make a reasonable compromise between experimental convenience (and hence better throughput) and a fair representation of steady state rates. If this idea is implemented in a microtiter plate format, with standard colormetric detection of reducing ends, it would be possible to produce about a dozen Michaelis Menten curves (either conventional or inverse) in one standard 96-well plate within a couple of hours and proportionate more if multiple plates are run in parallel. While certainly not high throughput by modern standards, this should be enough to sustain comparative analyses of cellulase function, based on *e.g.* the four kinetic parameters discussed here, using standard laboratory equipment. We emphasize that the simple kinetic approach presented here may not work for other types of cellulases such as endoglucanases, which produce both soluble and insoluble products. Hence, we advise to calibrate the method before using it to characterize cellulases with another mode of action then the one investigated here.

5. Conclusions

Cellulases show complex, multi-step reaction mechanisms that are difficult to investigate experimentally, and this is a challenge both for the scientific understanding and industrial utilization of these enzymes. One of the main obstacle for systematic and comparative cellulase kinetics is a shortage of quantitative assays and straightforward models for the interpretation of kinetic data. In the current study we have addressed these challenges for two of the best characterized cellobiohydrolases namely Cel7A and Cel6A from *Trichoderma reseei*. Using a large set of progress curves covering both low and high enzyme to substrate ratio we identified a time-domain where a reasonable descriptor of the quasi steady state rates could be obtained from the slope of tangents to the progress curve. We used these steady-state rates to obtain kinetic parameters by fitting the rates to simple Michaelis-Menten like equations. The parameters were compared with parameters obtained using a much simpler approach, where steady-state rates were estimated from 1 h end-point experiments (*i.e.* the slope of the secant). The result showed that the easier secant protocol gave a fair representation of kinetic parameters despite the experimental simplicity. We conclude that while rigorous determination of reaction rates must rely on the tangent method this approach is experimentally demanding and this drawback may well preclude its application in many cases. The secant method, on the other hand, provides a manageable compromise, which may become practical in biochemical characterization of cellulases based on steady state kinetics.

Acknowledgements

This work was supported by the Novo Nordisk Foundation, Innovation Fund Denmark (Grant no. 0603-00496B) and the Carlsberg Foundation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ab.2019.113411.

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