Cellobionic acid inhibition of cellobiohydrolase I and cellobiose dehydrogenase

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Abstract:

End-product inhibition by cellobiose and glucose is a rate-limiting factor in cellulose hydrolysis by cellulases. While cellobiose and glucose inhibition have been extensively investigated, cellobionate inhibition has been minimally studied despite the discovery that accessory proteins such as cellobiose dehydrogenase (CDH) work synergistically with cellulases to form aldonic acids. The fraction of equilibrated cellobionate in the cellobiono- δ -lactone form was determined by NMR over the range of pH 4 to 8. To better improve the understanding of cellobiono- δ -lactone and cellobionate inhibition of cellulases, we investigate the inhibition of *Trichoderma reesei* cellobiohydrolase I (CBHI), one of the most extensively studied CBHs, and *Neurospora*

crassa CDH by equilibrated cellobionate. The well-characterized inhibitor cellobiose is evaluated in parallel for comparison. The mechanisms of inhibition are determined for both inhibitors and the kinetic parameters compared using a Michaelis-Menten inhibition model. Cellobiose and cellobionate inhibition of CBHI is extended to Avicel hydrolysis, along with an investigation of those inhibitors on Accelerase 1500, a commercial cellulase preparation.

Introduction:

Due to its low cost and widespread abundance, lignocellulosic biomass is an attractive resource for renewable fuel and chemical production [1,2]. The primary obstacle to commercialization is the lack of a low cost processing technology, mainly due to the recalcitrance of cellulose. The steps involved in overcoming the recalcitrance (pretreatment, cellulase production, and enzymatic hydrolysis) account for approximately 40% of the total processing costs [3,4].

Hydrolysis of cellulose is achieved by a cellulolytic system of enzymes comprised of three main classes of enzymes: cellobiohydrolases (CBHs), endoglucanases (EGs), and β -glucosidases (BGLs). EGs hydrolyze cellulose internally, while CBHs hydrolyze cellulose at the reducing and non-reducing ends, liberating cellobiose in the process. BGLs hydrolyze cellobiose to glucose, which can be fermented to fuels and chemicals. End-product inhibition by cellobiose and glucose is a rate-limiting factor, with cellobiose being the stronger inhibitor, and remains a primary technical challenge in the process of enzymatic hydrolysis [5–8]. Relieving inhibition provides an opportunity to significantly improve cellulose hydrolysis rates and the overall processing cost.

One way to relieve end-product inhibition is to utilize cellobiose dehydrogenase (CDH), which is an important enzyme produced along with cellulases by some cellulolytic

microorganisms and catalyzes the oxidization of cellobiose to cellobionic acid. With the enzymatic oxidation of cellobiose via CDH, cellobiono- δ -lactone is formed initially [9,10]. However, cellobiono- δ -lactone is thermodynamically unfavorable with increasing pH in aqueous solution and spontaneously hydrolyzes to cellobionate. Furthermore, many cellulolytic fungi secrete lactonases which quickly hydrolyze cellobiono- δ -lactone to cellobionate [11,12]. It is speculated that the biological roles of CDH are to prevent product inhibition during cellulose degradation by oxidizing cellobiose, and to produce hydroxyl radicals through a Fenton-type reaction that can initiate depolymerization in lignin degradation [9,13,14]. Recently, the role of CDH was modified with the discovery that it works synergistically with polysaccharide monooxygenase accessory proteins (glycoside hydrolase 61 protein family, GH61) in oxidative cellulose degradation [15–18]. In our lab, we have developed a novel process which combines the cellulase production and enzymatic hydrolysis into a single step, and produces oxidized cellobiose (cellobionate) as the final hydrolysis product directly from cellulose. The engineered *N. crassa* strain with multiple copies of BGLs deleted contains all of the cellulase enzymes necessary for cellulose hydrolysis to cellobiose, with CDH oxidization of cellobiose to cellobionate [19,20]. Studying the inhibition of cellulases and CDH by cellobionate is useful for optimizing the system [21]. While cellobiose inhibition has been extensively investigated, cellobionate inhibition has been minimally studied. A previous study showed that cellobiono- δ -lactone inhibits total cellulase activity to a greater extent than the same concentration of cellobiose, whereas cellobionate has a weaker inhibition compared to cellobiose [18]. That same study showed that cellobionate does not inhibit BGL activity, but does not investigate cellobionate inhibition of the other individual cellulase components (CBH, EG, or CDH). Another study evaluates the kinetics of lactobiono- δ -lactone and lactobionate inhibition of CDH

with lactose as the substrate, concluding that the lactone is more inhibitory than the practically non-inhibitory acid form [12]. Currently, no kinetic information is available in the literature for cellobiono- δ -lactone or cellobionate inhibition of CDH. The K_m value of CDH for lactose is approximately 20-fold higher than the K_m value for cellobiose [12], making it difficult to infer that product inhibition of lactose is kinetically similar to product inhibition of cellobiose. To better improve the understanding of cellobiono- δ -lactone and cellobionate inhibition of cellulases, we investigate the inhibition of *Trichoderma reesei* CBHI, one of the most extensively studied CBHs, and the *N. crassa* CDH. The well-studied cellobiose inhibitor is characterized in parallel for comparison. The mechanisms of inhibition are determined for both inhibitors and the kinetic parameters compared using a Michaelis-Menten inhibition model. The inhibition of CBHI by cellobiose or cellobionate using Avicel as a substrate is also evaluated, as well as the inhibition of Accelerase 1500, a commercial cellulase cocktail. To our knowledge, this study provides the first direct comparison of cellobiose and cellobionate inhibition of CBHI and the first kinetic evaluation of cellobionate inhibition of CDH.

Materials and Methods:

Reagents:

CBHI from *T. reesei*, *p*-nitrophenyl- β -D-lactopyranoside (pNPL), and cellobiose were obtained from Sigma Aldrich (St. Louis, MO). CDH was obtained by a recombinant *Pichia pastoris* strain expressing CDHI from *N. crassa* as described previously [22]. Cellobionate was synthesized in our laboratory according to a previously established method as follows [23]. 3.8 g of cellobiose was dissolved in 20 mL of deionized water and heated at 55 °C for 10 minutes with stirring until completely dissolved. The solution was added to 50 mL of methanol. A solution of 5.7 g of iodine in 5 mL of methanol was added to the cellobiose solution and heated at 40 °C for 15

minutes with stirring. At room temperature and with stirring, 100 mL of 4% (w/v) potassium hydroxide-methanol solution was slowly added to the cellobiose-iodine solution, forming a sticky yellow precipitate. Another 100 mL of 4% (w/v) potassium hydroxide-methanol solution was added slowly, and the solution was stirred for an additional 30 minutes at room temperature. The cloudy top solution containing un-converted cellobiose was decanted from the yellow precipitate. The precipitate was dissolved in 5 mL of deionized water, to which another 100 mL of methanol was added. A white precipitate forms and settles quickly. Methanol is removed by centrifugation (6000g, 20 minute) and decanted. The potassium cellobionate precipitate was washed with 50 mL of methanol (three times) and tested for purity by HPLC.

NMR analysis:

^1H NMR spectra were collected at 35 °C using the standard 90° pulse and acquire sequence on an 800 MHz Bruker Avance III spectrometer equipped with a 5 mm Bruker HCN triple-resonance cryoprobe. NMR samples contained 5 mM cellobionate in buffer and 99.96% D_2O and were allowed to equilibrate for 72 hours prior to analysis. For pH 4 and 5, 10 mM sodium citrate buffer was used. For pH 6, 7, and 8, 50 mM sodium phosphate buffer was used. The desired pD was confirmed by a Metler Toledo glass pH meter, with the pH reading converted to pD by adding 0.4 [24]. NMR acquisition parameters were as follows: 32 scan averages, 11 ppm spectral width centered about the HOD resonance, 61618 points collected, 3.5 second acquisition time and a 3 second recycle delay for a total of 6.5 seconds repetition rate. The raw free-induction decay (FID) was multiplied by a 0.1 Hz exponential line broadening function before Fourier transform. Each spectrum was phased and baseline corrected, and chemical shifts were referenced by setting the internal anomeric hydrogen of α -cellobiose to 5.23 ppm [10]. Peak integrations were used to measure the cellobiono- δ -lactone to cellobionate ratios.

Inhibition studies:

Inhibition effects of cellobiose and cellobionate on CBHI were evaluated using pNPL as a substrate in a previously established enzymatic assay [25]. Final concentrations of reactants were 2.2 mM (1 mg/mL) pNPL, 0.05 U/mL of CBHI, and 0 – 5 mM of inhibitor in a total volume of 180 μ L. Solutions containing cellobiose or cellobionate inhibitors were allowed to equilibrate for at least 48 hours prior to analysis. The enzymatic reaction was initiated by the addition of enzyme and stopped after 15 minutes by the addition of 120 μ L of 1 M NaOH. The absorbance at 405 nm was compared to a standard curve of p-nitrophenol to determine the rate of release of p-nitrophenol. One unit of enzyme activity corresponds to the release of 1 μ mol of p-nitrophenol per minute. The assay was carried out in 50 mM sodium citrate buffer at pH 4 or pH 6 to determine the effect of the presence or absence of the lactone.

The analysis was repeated using an Accelerase 1500 cellulase cocktail at a final concentration of 0.05 U/mL (activity determined on pNPL according to the assay conditions described above).

The assay was carried out in 50 mM sodium citrate buffer at pH 5, the standard assay pH according to the previously established method [25].

The inhibition effect of cellobionate on CDH was evaluated using cellobiose as a substrate. Final concentrations of reactants were 0.015 U/mL CDH, 30 mM cellobiose, 0.3 mM 2,6-dichlorophenolindophenol (DCPIP) and 0 - 20 mM of cellobionate in a total volume of 200 μ L. CDH activity was determined by following the decrease in absorbance of DCPIP at 520 nm, as described previously, with one unit of enzyme activity corresponding to the reduction of 1 μ mol of DCPIP per minute [26]. The assay was conducted in 100 mM sodium citrate buffer at pH 4 or pH 6 to determine the effect of the presence of the lactone.

Kinetic analysis:

To determine the mechanism and kinetic parameters of cellobiose and cellobionate inhibition of CBHI, velocity versus substrate curves were generated for CBHI with various concentrations of pNPL (0.05 mM – 4 mM) and various inhibitor concentrations resulting in 20-80% inhibition (0.1 – 0.5 mM for cellobiose and 0.5 – 2.5 mM for cellobionate). The assay was performed in 50 mM sodium citrate buffer at pH 5. Assay conditions were maintained so that the total p-nitrophenol released was less than 5% of the initial substrate concentration. Lineweaver-Burk plots were generated to determine the mechanism of inhibition. The kinetic parameters were determined using non-linear regression (PolyMath), fitting the data to the appropriate Michaelis-Menten equation describing the mechanism observed from the Lineweaver-Burk plot. The mechanism and kinetic parameters of cellobionate inhibition of CDH were similarly determined, with the cellobiose substrate ranging from 0.05 - 10 mM and inhibitor concentration ranging from 0 – 10 mM. For kinetic analysis of CDH, the assay was conducted at pH 5 in 100 mM sodium acetate buffer.

Inhibition of equilibrated cellobionate solution on CBHI using Avicel as the substrate:

Hydrolysis reactions contained 50 mM sodium citrate buffer (pH 5.0), 20 g/L Avicel, and 10 mg/mL CBHI. The extent of hydrolysis with and without 2.5 mM of inhibitor (cellobiose or cellobionate) was evaluated by monitoring the release of reducing sugars using the dinitrosalicylic acid (DNS) method [27]. Samples were taken at various time points and centrifuged to remove unhydrolyzed Avicel. After 15 minutes of incubation at 37 °C, DNS reagent (0.01 g DNS, 3 g sodium potassium tartrate, 4 mL 1 M NaOH, with a total volume of 10 µL with DI water) was added to the sample and incubated in a thermocycler for 5 minutes at 95 °C, followed by 1 minute at 4 °C. The absorbance was measured at 540 nm and compared to

glucose standards (0 - 1.0 g/L). One unit of activity corresponds to the release of 1 μ mol of reducing sugar per minute.

Results:

We explore the inhibition effects of equilibrated mixtures of cellobionate on CBHI and CDH activity. In studies involving CBHI, cellobiose is also evaluated as a point of comparison. The mechanisms of inhibition of the equilibrated mixtures on both enzymes are evaluated and the kinetic parameters determined using a Michaelis-Menten model.

NMR analysis:

Equilibrium mixtures of cellobionate were analyzed by NMR to determine the relative ratios of cellobiono- δ -lactone and acid in the mixture at different pH. The chemical structures of the lactone and acid forms are given in Figure 1, and the NMR spectra for the pH range tested is shown in Figure 2. The protons corresponding to the relevant peaks are coded with a colored symbol as indicated in Figures 1 and 2. As determined previously, the chemical shifts at 4.58 ppm and 4.28 ppm correspond to the internal anomeric proton (red square) and C-2 proton (purple circle) of cellobiono- δ -lactone, respectively [10]. The chemical shift for the internal anomeric proton (blue diamond) of cellobionate is shifted up field to 4.63 ppm. The chemical shift for the C-2 proton (green triangle) of cellobionate ranges from 4.29 - 4.15 ppm, depending on the ratio of protonated to unprotonated acid as dictated by the pH [10]. The relative ratios of cellobiono- δ -lactone and cellobionate can be determined by comparing the peak areas of a given proton [28], as given in Table 1. The calculated relative amounts of cellobiono- δ -lactone and cellobionic acid align with those determined for glucono- δ -lactone and gluconic acid [29,30], indicating that above pH 5 the equilibrium tends to shift toward aldonic acids [15].

Inhibition of cellulases by cellobiose and cellobionate:

It is important to understand the effect of equilibrated cellobionate mixtures on cellulases in order to develop appropriate end-product inhibition relief strategies. Figure 3 shows the inhibition of CBHI by cellobiose or cellobionate using pNPL as a model substrate. Figure 3A shows that the extent of inhibition of CBHI by cellobionate is similar at pH 4 where the lactone form constitutes 5.4% of the equilibrated solution compared to pH 6 where only the cellobionate form exists. The data suggest that the small amount of lactone present does not significantly impact the results. Figure 3B shows the inhibition of CBHI by cellobiose. CBHI is inhibited to a greater extent by cellobiose compared to the same concentration of an equilibrated solution of cellobionate. Contrary to inhibition of CBHI, inhibition of CDH by an equilibrated solution of cellobionate at pH 4 and pH 6 show drastically differing results (Figure 3C). At pH 6, no inhibition is observed for cellobionate concentrations up to 20 mM. In contrast, at pH 4 where lactone is present, CDH is inhibited. This data suggests that the inhibition observed at pH 4 is due to the inhibitory effect of the lactone. The inhibition effects of cellobiose and cellobionate on Accelerase 1500, a commercial preparation of *T. reesei* cellulases, were subsequently evaluated (Figure 3D). The results again show decreased inhibition for cellobionate compared to cellobiose. Accelerase 1500 was less inhibited for a given concentration of cellobiose or cellobionate compared to purified *T. reesei* CBHI.

Next, the evaluation of cellobionate as an inhibitor was extended to Avicel, a real cellulosic substrate, for comparison to the model pNPL substrate. The hydrolysis of Avicel with CBHI with and without 2.5 mM of inhibitor (cellobiose or cellobionate) is shown in Figure 4. Avicel is hydrolyzed to a greater extent without either inhibitor present. The presence of 2.5 mM of

cellobionate inhibits Avicel hydrolysis to a lesser extent than the same concentration of cellobiose, a result that agrees with the inhibition observed with pNPL as a substrate.

Determination of kinetic parameters:

To determine the mechanism of inhibition, the reaction velocity was determined for various concentrations of inhibitor and substrate for CDH and CBHI. The double-reciprocal plots of the data, shown in Figure 5, depict a typical competitive inhibition mechanism for Michaelis-Menten kinetics. Based on the competitive inhibition Michaelis-Menten model (Equation 1), the kinetic parameters were calculated by non-linear regression and are listed in Table 2, where V_{max} is the maximum reaction rate in mM/min, S is the substrate concentration in mM, K_m is the Michaelis-Menten constant in mM, I is the inhibitor concentration in mM, and K_i is the inhibition constant in mM.

Equation 1

$$v = \frac{V_{max} * S}{K_m \left(1 + \frac{I}{K_i}\right) + S}$$

The K_m value of CBHI on pNPL was 1.05 mM. The K_i values of cellobiose and cellobionate inhibition of CBHI were 0.05 mM and 1.18 mM, respectively, indicating a 23-fold stronger inhibition by cellobiose than by cellobionate. The K_m value of CDH with cellobiose as a substrate was 0.172 mM, and the K_i value of cellobionate inhibition of CDH was 0.732 mM.

Discussion:

In this study, we seek to understand the inhibition effects of an equilibrated cellobionate solution on cellulases. Inhibition of CBHI by cellobionate was compared to inhibition by cellobiose. It was found that cellobionate is less inhibitory than cellobiose for CBHI, as well as the commercial cellulase preparation Accelerase 1500, when pNPL is used as a substrate. Similar

results were found for CBHI hydrolysis of Avicel. Inhibition of CDH by cellobionate was also evaluated. No inhibition was observed when only the acid form was present, whereas an equilibrated mixture of cellobionate containing the lactone form does show inhibition. Inhibition of both CBHI and CDH were modeled according to Michaelis-Menten kinetics.

The NMR data presented here show that equilibrated cellobionate contains approximately 5.4% cellobiono- δ -lactone at pH 4. In contrast, at pH 6 an equilibrated cellobionate solution contains only the acid form. At pH 5, which is the optimal pH for cellulases, the equilibrated cellobionate contains approximately 1.9% cellobiono- δ -lactone. Obvious inhibition of CBHI by cellobionate at pH 6 was observed, which indicates that the cellobionate is inhibitory to CBHI activity in 100% acid form. Inhibition of *T. reesei* CBHI by a cellobionate solution at pH 4 shows a similar degree of inhibition to a cellobionate solution at pH 6, implying that the presence of a small fraction of cellobiono- δ -lactone does not change the extent of inhibition of the cellobionate solution on CBHI activity significantly.

In parallel experiments, cellobiose inhibition on CBHI was tested at the same concentration as cellobionate and showed much stronger inhibition. This result aligns with a previous study evaluating the effects of hydrolysis products on cellulase activity as a whole, which showed that cellobiose had a stronger inhibitory effect compared to cellobionate [18]. Additionally, the extent of inhibition of CBHI by cellobiose is similar at pH 4 and pH 6.

When Accelerase 1500, a commercial cellulase cocktail, is evaluated, cellobiose again shows stronger inhibition compared to cellobionate for a given inhibitor concentration. However, in contrast to purified CBHI, the magnitude of inhibition by either cellobiose or cellobionate decreases when the cellulase cocktail is used. This result is not surprising, since cellulase cocktails contain a variety of enzymes, including CBHIs, CBHIIIs, EGs, and BGLs. Previous

studies have shown that CBHI enzymes are inhibited by cellobiose to a greater extent than other cellulases [5–7]. Lastly, when cellobionate inhibition is compared to cellobiose inhibition using Avicel as a substrate, the rate and extent of hydrolysis is less impacted by the presence of cellobionate than by the presence of cellobiose, a result similar to that obtained when pNPL is used as the substrate.

To elucidate the mechanism of inhibition, the reaction rate was calculated for several substrate and inhibitor concentrations and double-reciprocal plots were generated as shown in Figure 5. The double-reciprocal plots depict a competitive inhibition mechanism. The data was fit to the Michaelis-Menten equation for competitive inhibition (Equation 1) using non-linear regression to determine the kinetic parameters, as shown in Table 2. The calculated K_m value aligns with previous results in literature for pNPL and *T. reesei* CBHI [25], and the mechanism of inhibition and calculated K_i value for cellobiose aligns with a similar study with the CBHI from *Phanerochaete chrysosporum* [31]. The K_i value for cellobionate inhibition of CBHI was 23 times higher than that of cellobiose, indicating a much weaker inhibition effect. This result is supported by a study showing that cellobiose inhibition of CBHI by *P. chrysosporium* is relieved in the presence of CDH and a redox mediator, which would convert the cellobiose to cellobiono- δ -lactone and cellobionate [31]. However, the same study shows that inhibition of CBHI from *T. viride* increases in the presence of CDH and a redox mediator. It appears that the effects of cellobiono- δ -lactone and cellobionate on CBHI is dependent on the source of CBHI.

In contrast to cellobionate inhibition of CBHI from *T. reesei*, CDH from *N. crassa* does not appear to be inhibited by cellobionate. At pH 6, where cellobionate is present in only its acid form, no inhibition is observed for concentrations up to 20 mM. At pH 4, where 5.4% of the cellobionate solution is in the lactone form, inhibition is observed. These results suggest that the

cellobiono- δ -lactone is significantly more inhibitory on CDH than cellobionate, which is quite different than its effect on CBHI. This result is similar to a previous study evaluating the effects of lactobionate and lactobiono- δ -lactone inhibition of CDH when lactose is used as a substrate [12]. It is also important to note that the K_m value of CDH with lactose is 2.4 mM at 25 °C, compared to 0.12 mM when cellobiose is the substrate [12]. Our calculated K_m value of 0.17 mM at 30 °C aligns well with the literature values of 0.12 mM at 25 °C and 0.28 mM at 45 °C [12]. While the acid form of cellobionate does not have an inhibitory effect on CDH, our kinetic studies show that an equilibrated cellobionate solution (where a portion is in the lactone form), has a notable inhibitory effect. The calculated K_i value for an equilibrated cellobionate solution is 0.73 mM. This indicates a much stronger inhibition as compared to an equilibrated lactobionate solution, where the authors report the K_i values for an equilibrated lactobionic acid solution, and a calcium lactobionate solution to be 510 mM and 1600 mM, respectively [12]. These values are much higher than the K_i value we find for an equilibrated cellobionate solution on CDH using cellobiose as the substrate. It is difficult to draw comparisons since the cited study has an equilibrated mixture of lactobionic acid that contains 84% acid and 16% lactone, which is quite different than our relative ratio. In addition, they characterized a CDH from a different microorganism, which may have a different affinity to substrates and inhibitors.

In summary, this study shows that cellobionate inhibition is weaker than cellobiose inhibition for purified CBHI or a commercial cellulase blend using the model pNPL substrate, as well as for purified CBHI using Avicel as a substrate. Our data show that the K_i value for cellobionate is approximately 23 times higher than that for cellobiose for CBHI from *T. reesei*, indicating a much weaker inhibition effect. Since *T. reesei*'s CBHI is considered one of the workhorse enzymes for cellulose hydrolysis, and CBHs are the class of cellulases most strongly

inhibited by cellobiose [5–7], cellobiose inhibition relief strategies are important for improving the overall rate of enzymatic conversion of cellulose [32]. Our data show that oxidation of cellobiose to cellobionate will likely improve conversion rates through such inhibition relief. It is noteworthy that cellobionate can be an alternative substrate for fuels and chemicals production via fermentation. Ethanol production from cellobionate can be achieved by engineering *E. coli* to express BGL or by adding exogenous BGL to hydrolyze cellobionate into equimolar amounts of glucose and gluconate. Both of the hydrolysis products from cellobionate, glucose and gluconate, can be used efficiently by *E. coli* KO11 to produce ethanol and acetic acid [33,34]. Alternatively, *E. coli* can directly consume cellobionate as the carbon source for isobutanol production; *Saccharomyces cerevisiae* has been engineered to use cellobionate for ethanol production [21,35]. Furthermore, equilibrated solutions of cellobionate containing cellobiono- δ -lactone have significant inhibition of CDH, highlighting the importance of lactonases in whole cellulolytic systems [11,18]. The extent to which cellulases other than CBHI and CDH are inhibited by cellobionate is beyond the scope of this study. However, the fact that CBHIs are considered the workhorse enzyme of cellulases suggests that relieving inhibition of this enzyme can potentially improve hydrolysis rates of cellulose for the complete cellulolytic system. In support of this hypothesis, we show that Accellerase 1500, a commercial cellulase preparation, is inhibited to a lesser extent by cellobionate than by cellobiose.

Conclusions

This study describes the inhibitory effect of equilibrated cellobionate on cellulases. Compared to cellobiose, equilibrated cellobionate is less inhibitory for CBHI and Accelerase 1500 when pNPL is used as a substrate. Similarly, Avicel hydrolysis by CBHI is inhibited to a lesser extent by equilibrated cellobionate compared to inhibition by cellobiose. CDH is inhibited when the

lactone form is present in the equilibrated cellobionate solution. No inhibition of CDH is observed when only the acid form is present. While additional studies can further elucidate the mechanisms behind inhibition reduction, this study demonstrates the potential of converting cellobiose to cellobionate to partially relieve product inhibition by cellobiose and provides the motivation for continued research in this area.

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Figure captions:

Figure 1. Chemical structures of cellobiono- δ -lactone and cellobionate, with relevant protons for the NMR analysis identified by color-coded symbols. Red square = internal anomeric proton for cellobiono- δ -lactone; purple circle = C-2 proton for cellobiono- δ -lactone; blue diamond = internal anomeric proton for cellobionate; green triangle = C-2 proton for cellobionate. Figure adapted from [36].

Figure 2. Proton NMR spectra for cellobionic acid and cellobiono- δ -lactone at various pH. Figure 2A shows the full spectra at varying pH. Figure 2B is the spectra zoomed in at 4.58 ppm, corresponding to the internal anomeric proton of cellobiono- δ -lactone. Figure 2C is the spectra zoomed in at 4.28 ppm, corresponding to the C-2 proton of cellobiono- δ -lactone. The green triangles and blue diamond correspond to the C-2 proton and internal anomeric proton of cellobionate, respectively.

Figure 3. A) cellobionate inhibition of CBHI at pH 4 or 6; B) cellobiose inhibition of CBHI at pH 4 or 6; C) cellobionate inhibition of CDH at pH 4 or 6; D) cellobiose or cellobionate inhibition of Accelerase 1500 at pH 5. At pH 4, the equilibrated solution of cellobionate is approximately 5.4% cellobiono- δ -lactone and 94.6% cellobionate. At pH 6, the equilibrated solution of cellobionate is entirely in the acid form. Error bars represent the standard deviation of reaction triplicates.

Figure 4. Avicel hydrolysis by CBHI with and without inhibitors present. Error bars are the standard deviations of triplicate measurements.

Figure 5. Double-reciprocal plots showing competitive inhibition of CBHI with pNPL as the substrate for A) cellobionate and B) cellobiose, and C) cellobionate inhibition of CDH with

cellobiose as the substrate. D) Kinetic constants, given in mM, of CBHI inhibition by cellobiose and cellobionate and CDH inhibition by cellobionate.

Table 1. Relative percent of cellobiono- δ -lactone and cellobionic acid. The percentage was calculated based on the ratio of the peak areas for the C-2 protons and internal anomeric protons, as determined from the spectra in Figure 2. Standard deviations are from duplicate measurements.

RELATIVE %		
	<i>cellobiono-δ-lactone</i>	<i>cellobionic acid</i>
pH 4	5.4 $\pm$ 0.2	94.6 $\pm$ 0.2
pH 5	1.9 $\pm$ 0.2	98.1 $\pm$ 0.2
pH 6	0.0 $\pm$ 0.0	100.0 $\pm$ 0.0
pH 7	0.0 $\pm$ 0.0	100.0 $\pm$ 0.0
pH 8	0.0 $\pm$ 0.0	100.0 $\pm$ 0.0

Table 2. Kinetic constants, given in mM, of CBHI inhibition by cellobiose and cellobionate at pH 5 with pNPL as the substrate, and CDH inhibition by cellobionate at pH 5 with cellobiose as the substrate.

CBHI kinetics			
<i>Parameter</i>	<i>value</i>	<i>95% confidence interval</i>	<i>R²</i>
K _m	1.003	4.79E-02	0.998
K _i - cellobiose	0.052	5.50E-06	0.992
K _i – cellobionate, equilibrated	1.184	2.38E-05	0.981
CDH kinetics			
<i>Parameter</i>	<i>value</i>	<i>95% confidence interval</i>	<i>R²</i>

K_m	0.172	2.90E-02	0.932
$K_i - \text{cellobionate, equilibrated}$	0.732	1.97E-04	0.941

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Relative Percentage of equilibrate cellobionate solution

	<i>cellobiono-δ-lactone</i>	<i>cellobionic acid</i>
pH 4	5.4 ± 0.2	94.6 ± 0.2
pH 6	0.0 ± 0.0	100.0 ± 0.0