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Effects of Sugar Inhibition on Cellulases and β-Glucosidase During Enzymatic Hydrolysis of Softwood Substrates

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Abstract

A quantitative approach was taken to determine the inhibition effects of glucose and other sugar monomers during cellulase and β-Glucosidase hydrolysis of two types of cellulosic material: Avicel and acetic acid–pretreated softwood. The increased glucose content in the hydrolysate resulted in a dramatic increase in the degrees of inhibition on both β-Glucosidase and cellulase activities. Supplementation of mannose, xylose, and galactose during cellobiose hydrolysis did not show any inhibitory effects on β-Glucosidase activity. However, these sugars were shown to have significant inhibitory effects on cellulase activity during cellulose hydrolysis. Our study suggests that high-substrate consistency hydrolysis with supplementation of hemicellulose is likely to be a practical solution to minimizing end-product inhibition effects while producing hydrolysate with high glucose concentration.

Index Entries: β-Glucosidase; cellulase; degree of inhibition; softwood; glucose; hydrolysate.

Introduction

The utilization of renewable lignocellulosic resource, such as wood and agricultural residues, for ethanol production provides a promising means to decrease the greenhouse effect and alleviate the shortage of fossil fuel energy *(1,2)*. An integrated lignocellulose-to-ethanol biocon-

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version process consists of at least the following steps: removal of lignin and hemicellulose by pretreatment, hydrolysis of cellulose to fermentable sugars, and fermentation of sugars to ethanol. The hydrolysis of cellulose to glucose has been shown to be the key step in the bioconversion of lignocellulosic biomass. Cellulose hydrolysis can be carried out by either acidic or enzymatic treatment. The maximum yields of glucose obtained from batch and bed-shrinking flow-through reactors, through acidic treatment, were about 60 and 90%, respectively, for α-cellulose *(3)*. The enzymatic process converts cellulose to glucose in high yields without sugar degradation products and has been recognized as the method of choice for future wood-to-ethanol processes *(4,5)*. However, currently, there are some limitations associated with cellulase enzymes that hinder the application of enzyme at industrial scale *(6–8)*. Unlike many other enzymes, cellulase is not a single enzyme but a family of at least three groups of enzymes: endoglucanase (EC 3.2.1.4), cellobiohydrolase (CBH) (EC 3.2.1.91), and β-Glucosidase (EC 3.2.1.21). A synergistic action among these enzymes is required to effectively break down cellulose to glucose. A typical cellulose hydrolysis pattern in a batch mode enzymatic process is characterized by a two-phase curve, with an initial logarithmic phase followed by an asymptotic phase *(9)*. A rapid release of glucose is normally observed in the initial phase with about half of the cellulose hydrolyzed in less than 24 h. On the other hand, the hydrolysis of the remaining cellulose requires more than 2 days to complete *(10)*. Several mechanisms have been proposed for this insufficient hydrolysis phenomenon *(11–13)*. However, end-product inhibition has been shown to play a major role in hindering a continuously fast hydrolysis rate *(14)*, and glucose, cellobiose, and ethanol have demonstrated significant inhibitory effects on the activity of both β-glucosidase and cellulase mixtures *(8)*.

A considerable amount of research has been carried out to elucidate the mechanism of end-product inhibition on cellulase enzyme systems during cellulose hydrolysis *(6,8)*. However, there have been few attempts to quantitatively determine the degree of sugar inhibition on the cellulase enzymes during cellulose hydrolysis. Because of the intricate nature of lignocellulosic material, most of the mechanisms proposed for pure cellulose substrates, such as Avicel, and Solka Floc, are typically not representative of what happens on these never-dried substrates with various degrees of noncellulosic contaminants *(9)*. There is also a lack of information on whether monosaccharides other than glucose will have inhibitory effects on cellulase activity during cellulose hydrolysis.

In the present study, we quantified the degree of inhibition on both β-glucosidase and cellulase mixtures by glucose and cellobiose at different concentrations. We also determined the inhibitory effects of mannose, galactose, and xylose on both β-glucosidase and cellulase activities and assessed the potential to increase the final sugar concentration by supplementing cellulosic substrate hydrolysis with hemicellulose-rich stream-obtained from steam exploded softwood (prehydrolysate).

Materials and Methods

Enzymes and Hydrolysis

The complete cellulase system (Celluclast®) and β-glucosidase (Novozym 188®) used were obtained from Novo Nordisk Biochem (Franklinton, NC). The cellulolytic activities determined in the Celluclast preparation are 121 filter paper units (FPU)/mL, and 45 cellobiase units (CBU)/mL. The activities of Novozym 188 are 570 CBU/mL and 2.0 FPU/mL. The enzyme activities were measured according to standard procedures *(15)*. All the hydrolysis experiments were conducted in 500-mL Erlenmeyer flasks at 50°C using 50 mM sodium acetate buffer (pH 4.8) with shaking at 150 rpm. The substrate consistency during acetic acid–pretreated softwood hydrolysis is 10% and the enzyme loading is 40 FPU of Celluclast®/g of cellulose supplemented with 80 CBU of β -glucosidase.

The concentration of glucose released during the hydrolysis was measured using a YSI 2700 Select Biochemistry Analyzer (Yellow Springs Instruments, Yellow Springs, OH), and the concentrations of mannose, galactose, and xylose were measured by high-performance liquid chromatography *(16)*.

Substrates and Chemicals

The sugars used as inhibitors were glucose (Sigma, St. Louis, MO), mannose (Sigma), galactose (G-0750; Sigma), and xylose (Fisher Scientific, Fair Lawn, NJ). Avicel, cellobiose, *p*-nitrophenyl-β-D-glucopyranoside (pNPG) and all the sugar monomers were obtained from Sigma. Acetic acid-pretreated softwood substrate was prepared as described previously.

Removal of End Products During Acetic Acid–Pretreated Softwood Hydrolysis Through Ultrafiltration

After 24 h and 48 h of hydrolysis, the hydrolysate was separated from the residue solids by centrifuging at 11,950*g* for 10 min. The supernatant was then filtered through a 10-kDa membrane at 4°C in a 160-mL Amicon ultrafiltration unit (Beverly, MA). Fresh buffer was added during the filtration to remove the soluble end products. The retained enzymes in the Amicon unit were added back to the solid residues, and fresh buffer was added to the initial volume without any additional enzymes.

Determination of Degree of Inhibition Caused by Sugar Monomers

The degree of inhibition was based on the ratio of the hydrolysis rates with and without the presence of supplemented sugars, V_I/V., in which V_I is the amount of glucose (g) produced from the substrate in the presence of supplemented sugars/30 min, and V is the amount of glucose (g) produced from the substrate without sugar supplementation/30 min. The hydrolysis rates were determined after 30 min of hydrolysis. The short hydrolysis period was selected to minimize the inhibitory effects of the released sugars.

Results and Discussions

Significance of End-Product Inhibition on Hydrolysis of Lignocellulosic Substrates

In an earlier work, we showed that acetic acid pulping is an effective pretreatment method for converting softwoods. The pulped substrate contained very low amounts of lignin (~5%) and hemicellulose and showed a lower degree of polymerization and crystallinity in the cellulose when compared to the raw material. The acetic acid–pretreated softwood substrate showed a greater degree of hydrolyzability to cellulase, which was superior to many other cellulosic substrates including Avicel, and steam-exploded pulp This greater digestibility enables hydrolysis at higher substrate concentrations (10–20%).

A 72-h hydrolysis profile of a 10% acetic acid–pretreated softwood substrate (Fig. 1) represents a typical enzymatic cellulose hydrolysis course with the majority of the cellulose (up to 70%) broken down within the first 24 h. However, the conversion of the remaining cellulose (~30%) was incomplete, even after another 2 d of incubation. The decrease in the hydrolysis rate in the latter phase is likely owing to accumulation of end products. To demonstrate that the end products played a major inhibitory role, we removed the produced sugar from the hydrolysate through ultrafiltration. Fresh buffer was then added to the retained protein and the residual substrate to attain the initial volume, and the hydrolysis was continued under the same condition. As shown in Fig. 1, significant increases in the hydrolysis rate were observed after the sugar removal at both 24 h and 48 h of incubation, with complete hydrolysis attained after 48 h and 60 h of incubation respectively.

Enzymatic hydrolysis of cellulose can be separated into two major steps. The first step depolymerizes and hydrolyzes cellulose into soluble cellobiose, and the subsequent step converts cellobiose to glucose by β-glucosidase. Several cellulolytic enzymes are involved in the first hydrolysis step, and the β-glucosidases are the predominant group of enzymes that carry out the latter step of the conversion. As a final product, glucose has a direct inhibitory effect on β-glucosidase activity. Kinetic studies of glucose inhibition on β-glucosidase have been an area of debate *(8),* although the actual inhibition mechanism is largely dependent on the sources of enzymes *(7,17–19)*.

In the present study, rather than try to distinguish the nature of inhibition, we tried to determine the degree of inhibition on β-glucosidase activity by adding different amounts of glucose to the hydrolysate. The inhibitory effects of several other monosaccharides, including mannose, galactose, and xylose, were also determined. The degree of inhibition was expressed by the ratio between the hydrolysis rate in the presence of possible inhibitor (V_I) and hydrolysis rate of a control cellulose hydrolysis without any sugar supplementation (V). The hydrolysis rate was calculated based on the glucose released from the substrates, pNPG—cellobiose,

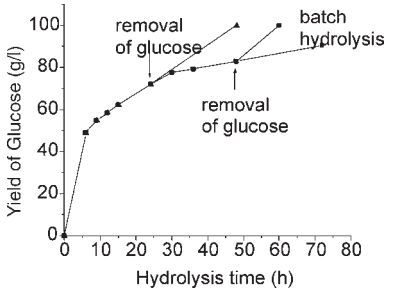

Fig. 1. Effects of glucose removal on the hydrolysis rate of acetic acid–pretreated wood.

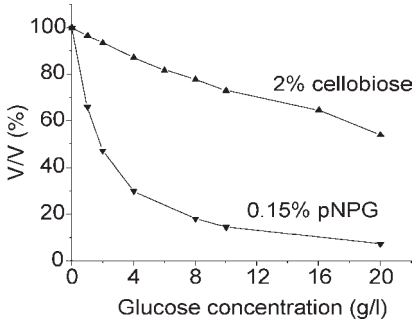

Fig. 2. Degree of Inhibition on β-glucosidase activity caused by addition of glucose during hydrolysis of cellobiose and pNPG.

Avicel, and acetic acid–pretreated softwood—after a 30-min incubation with respective enzymes.

Degree of Inhibition on β-glucosidase Activity by Different Amounts of Glucose

The degree of inhibition on β-glucosidase activity in the presence of glucose from 0 to 20 g/L was tested (Fig. 2) using two different types of substrate: pNPG and cellobiose. Although pNPG is a substrate analog to cellobiose, the pNPG assay provides a quick and simple method to determine the effect of supplemented glucose on β-glucosidase activity. However, because the measurement of sugar is based on colorimetry, only very low substrate (pNPG) concentrations, 5 m*M* (0.15% [w/v]), were used, whereas the concentration of cellobiose used in the hydrolysis was 2% (w/v). The β-glucosidase (Novozym188) loading was 80 CBU/g of the substrate, either pNPG or cellobiose. A significant inhibitory effect was observed on the hydrolysis rate even at very low glucose concentration when pNPG substrate was used (Fig. 2). The reaction rate was reduced by >80% when the glucose concentration exceeded 10 g/L. A steady decrease in the hydrolysis rate with increases in glucose concentration was also observed when cellobiose was subjected to β-glucosidase hydrolysis.

However, the degree of glucose inhibition at the same glucose concentration on the 2% cellobiose hydrolysis was less significant than that obtained on the pNPG. Since the ratio between the enzyme and the substrate is constant, higher substrate concentration means higher enzyme input. Thus, the reason for the reduced impact on inhibition, as the consistency increases, is probably owing to the higher enzyme-to-inhibitor ratio encountered in the cellobiose hydrolysis system compared with that in the pNPG hydrolysis system.

The degree of glucose inhibition on β-glucosidase activity at different substrate concentrations was further investigated. Three cellobiose consistencies were selected—2, 5, and 10%—along with a β-glucosidase loading of 80 CBU/g of cellobiose. The maximum amounts of glucose supplementation—20, 50, and 100 g/L—were chosen to correlate with the three different substrate consistencies at 2, 5, and 10%, respectively. Again, the effects of inhibition at the same glucose supplementation levels became less significant as the substrate concentration increased from 2 to 10% (Fig. 3). However, since the degree of inhibition was determined at the same enzyme-to-glucose ratio, all three experiments seemed to give similar results. The degrees of inhibition were 53, 51, and 48% correlated to 20, 50, and 100 g/L of glucose supplementation in 2, 5, and 10% cellobiose hydrolysates, respectively (Fig. 3).

Degree of Inhibition on Cellulase Activity by Different Amounts of Glucose

The degree of glucose inhibition on the cellulase activity was also determined using Avicel as a substrate. Two substrate concentrations, 2 and 10%, and glucose supplementation from 0 to 100 g/L were tested. The enzyme loading was 40 FPU (Celluclast) with 80 CBU (Novozym188)/g of cellulose. The addition of glucose resulted in significant inhibitory effects on the cellulase activity. The results agree with many previous findings *(12,20,21)*. It was interesting to find that at the same glucose supplementation, a higher degree of inhibition was observed on the cellulase activities than on the β-glucosidase (Fig. 4) at both substrate concentrations. As mentioned earlier, cellulase is a family of multiple enzymes, including at least endoglucanase, CBH, and β-glucosidase. This result seemed to indicate that the glucose may have a direct inhibitory effect on the cellulase enzymes other than just β-glucosidase. Previous research has shown that glucose has a significant impact on endoglucanase and CBH activity *(22,23)*. It has also been shown that cellobiose exhibits a greater impact than glucose on cellulase activity during cellulose hydrolysis *(6–8,24)*. It is probable that the high glucose content in the hydrolysate leads to the accumulation of cellobiose, which then acts as a secondary inhibitor. Therefore, the combined inhibitory effects have a greater impact on the overall cellulase activity than that of single sugar. Other studies have also shown that cellobiose is more inhibitory to the overall cellulase activity than is glucose *(25–27)*.

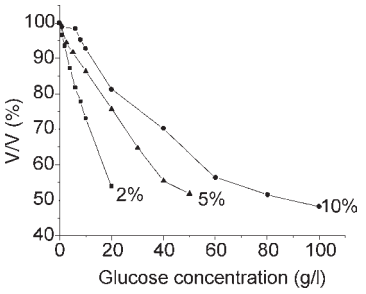

Fig. 3. Degree of Inhibition on β-glucosidase activity caused by the addition of glucose during hydrolysis of cellobiose at three different substrate concentrations: 2, 5, and 10% (w/v).

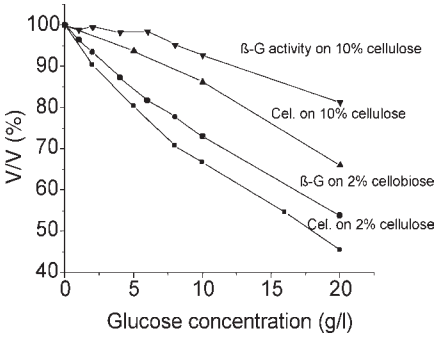

Fig. 4. Comparison of degree of glucose inhibition on cellulase and β-glucosidase activities during hydrolysis of cellulose and cellobiose at two substrate consistencies: 2 and 10% (w/v). Cel, cellulase; β-G, β-glucosidase.

Inhibitory Effects of Mannose, Galactose, and Xylose on β*-glucosidase*

Mannose, galactose, and xylose are the major monosaccharides formed in the hemicellulose-rich fraction of steam-exploded softwoods. Most of these sugars are present in the dilute water-soluble fraction after various pretreatment processes. Low ethanol concentration after the fermentation of this stream resulted in high costs associated with the subsequent evaporation and recovery process steps *(28,29)*. High sugar concentrations are desirable after enzymatic hydrolysis in order to minimize the cost of the overall bioconversion process. Cellulose hydrolysis supplemented with the hemicellulose-rich water-soluble stream obtained after steam explosion provides a potential way to enhance the final sugar concentration. Little information has been published on the inhibitory effect of hemicellulose-derived sugars on β-glucosidase and cellulase activities, although Dekker *(7)* reported a 13% decrease in β-glucosidase activity that was detected at 5% D-xylose supplementation.

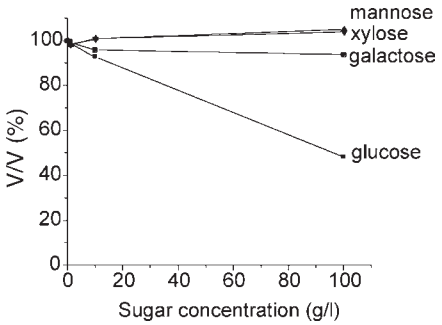

Fig. 5. Degrees of Inhibition on β-glucosidase activity caused by addition of mannose, galactose, and xylose.

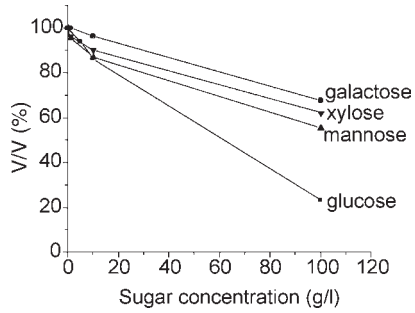

Fig. 6. Degrees of Inhibition on cellulase activity caused by addition of mannose, galactose, and xylose.

None of the monosaccharides in our study showed any observed inhibition on β-glucosidase at concentrations up to 100 g/L (Fig. 5). Other research has also shown that arabinose, galactose, mannose, and xylose were not inhibitory at concentrations up to 56 m*M (30,31)*. β-Glucosidase has been shown to be quite specific in its response to the nature of the inhibitors *(8,32)*, and β-glucosidases obtained from different origins have been shown to have different responses to these various inhibitors *(7,30,31)*.

Mannose, galactose, and xylose supplementation demonstrated significant inhibition of hydrolysis rate (Fig. 6). Hydrolysis rates decreased by 32, 38, and 45% after supplementation with galactose, xylose, and mannose, respectively, at 100 g/L. The hemicellulose-derived sugars seemed to have a direct inhibitory effect on the cellulase enzymes. The detailed inhibitory mechanisms of these sugars on the cellulase activities were not clear and are currently under investigation. However, it is likely that the mechanisms will depend on both the characteristics of the specific inhibitors and the structure of the different enzymes.

Inhibitory Effect of Synthetic Hemicellulose-Rich Water-Soluble Fraction on High-Cellulose Consistency Hydrolysis

Previous research indicated that the biomass-to-ethanol process could be more economically feasible by incorporating the hemicellulose-rich, water-soluble fraction with the enzymatic hydrolysis of the solid fraction *(14)*. We have shown that inhibitory effects caused by the hemicellulose-derived sugars were less significant than glucose. Our results also indicate that increases in substrate concentration during hydrolysis may reduce the degree of inhibition caused by the same amount of inhibitors. Since the water-soluble stream obtained after steam explosion of softwoods are typically low in sugar concentration (1–5%), we next examined the potential to enhance the effects of final sugar concentration by combining the cellulose hydrolysis stream with hemicellulose-rich water-soluble stream. A synthetic sugar mixture containing glucose, galactose, and mannose at a ratio of 1:1:3 was prepared. This mixture represents the predominant sugars derived from softwood hemicellulose, and the ratio reflects the composition of these sugars found in most softwood species *(33,34)*. Two different concentration levels, 1 and 2%, were used in combination with the 2% glucose stream.

Hydrolysis was carried out at 50°C for 48 h with an enzyme loading of 40 FPU + 80 CBU/g of cellulose. The substrate used was acetic acid pulp at a 10% substrate concentration. The yields of glucose released from the three sugar-supplemented hydrolysis samples were lower than that obtained with control sample (without any sugar addition) over the 48-h incubation time (Fig. 7). As expected, 20 g/L of glucose exhibited the strongest inhibition on the hydrolysis, followed by 20 g/L and 10 g/L of sugar supplementation in the hydrolysate. The degree of inhibition was measured over the hydrolysis period (Fig. 8), and it was apparent that the presence of 10 g/L and 20 g/L of hemicellulose sugars decreased the hydrolysis rate by 10–15% in the first hours of hydrolysis, and a 45% reduction in hydrolysis rate was observed for the sample supplemented with 20 g/L of glucose. However, as the hydrolysis proceeded, the three hydrolysis curves approached the control hydrolysis curve. After 48 hours of incubation, the rates of all four hydrolysis runs were comparable. This is probably because the inhibitory effects of the produced glucose surpassed the inhibitory effects of the supplemented sugars.

As mentioned earlier, the purpose of carrying out cellulose hydrolysis in the presence of hemicellulose-rich, water-soluble fraction was to enhance the overall sugar concentration in the final hydrolysate prior to the fermentation. When total sugar concentrations were also monitored during the 48-h hydrolysis (Fig. 9), although the supplemented sugars showed various degrees of inhibition on the hydrolysis, the total sugar concentrations present in the three sugar-supplemented hydrolysates after 48 h of hydrolysis were higher than that obtained with control. Total sugars concentrations of 101.8, 95.4, and 98.1 g/L were obtained, respectively, in the presence of the 2% hemicellulose-derived sugars, 1% hemicellulose-derived sugars, and

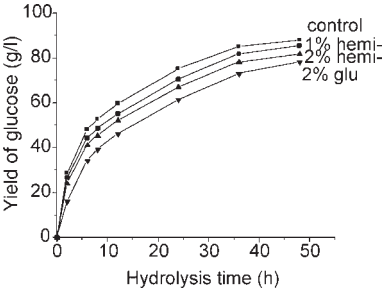

Fig. 7. Inhibitory effects of supplemented hemicellulose-derived sugars on hydrolysis of 10% acetic acid-pretreated softwood.

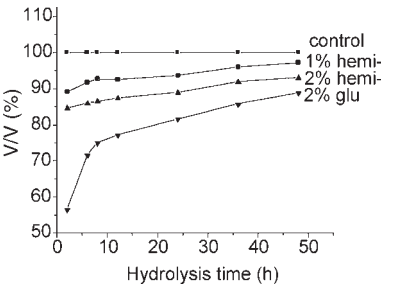

Fig. 8. Degree of Inhibition caused by supplemented hemicellulose-derived sugars on hydrolysis of 10% acetic acid-pretreated softwood.

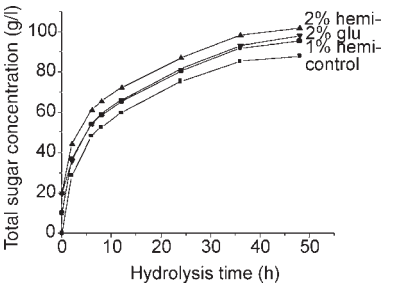

Fig. 9. Total sugar concentrations during combined hydrolysis (10% acetic acid–pretreated softwood substrate with prehydrolysate).

2% glucose-supplemented hydrolysate after 48 h of hydrolysis compared with 87.9 g/L obtained in the control hydrolysate.

Conclusions

The degree of inhibition of glucose on both β-glucosidase and cellulase activities was determined at glucose supplementation levels up to 100 g/L.

It was apparent that glucose had a greater inhibitory effect on the overall cellulase activity than it had on the β-glucosidase. Although the higher substrate concentration seemed to be able to alleviate some of the inhibitory effects at the same glucose concentrations, the degree of inhibition measured based on the same enzyme-to-inhibitor ratio was comparable. No adverse effects were detected on the β-glucosidase activity in the presence of mannose, galactose, and xylose at concentrations up to 100 g/L, and these sugars seemed to show some inhibition towards, the other cellulase components. Combining the cellulose hydrolysis with the hemicellulose sugars present in the water-soluble stream obtained from steam-exploded softwood was shown to be a promising strategy for enhancing the final sugar concentration. The results from our study suggest that high-substrate consistency hydrolysis with supplementation of hemicellulose could be a practical solution for minimizing end product-inhibition effects while producing a hydrolysate with high glucose concentration.

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