Rapid Discrimination of Endo- and Exo-cellulases

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ABSTRACT

Steady shear viscosities in the Newtonian range as a function of time after addition of exo- and endo-cellulases to 5 wt% solutions of carboxymethyl cellulose (CMC) were measured. The results show that exo-enzymes have little effect on solution viscosities, whereas endo-enzymes reduce viscosities effectively. The results are supplemented by biochemical end-group assays and size exclusion chromatography.

INTRODUCTION

There is a big interest in the production of biofuels on an industrial scale. Bioethanol can be used as transport fuel, it is more sustainable than fossil fuels, and it is CO₂ Biofuel produced neutral. is from biomaterial from either first or secondfeedstocks.¹ generation Enzymatic degradation of cellulose from both first and second generation feedstock is possible with enzymes, e.g. Cel6a and Cel7a from Trichoderma reesei. However, optimal degradation of cellulose into glucose requires a mixture of enzymes consisting of endoglucanase exocellulases, and ßglucosidase.¹ It is desirable to have a quick method investigate screening to and quantify endo-activity with newly discovered or newly genetically modified enzymes. There are a number of different methods to study the endo-activity of enzymes, however none of these quantify

the

activity.²

In this study the aim is to find a rapid and reliable way to screen enzymes for endoactivity and furthermore be able to quantify endoactivity of different cellulases. Carboxymethyl cellulose (CMC) is used as substrate to distinguish between endo- and exo-activity. Exo-cellulases are only able to hydrolyze non-substituted ends of CMC whereas endo-glucanases can hydrolyze at random non-substituted sugars along the CMC chains.³ The main advantage of using CMCs as a model system is the fact that they possess a high solubility in hydrophilic environments in contrast to native cellulose.⁴ Rheological properties of CMC solutions depend on the molecular weight, molecular structure the and the concentration of CMC. In a semi-dilute solution, the CMC molecules are entangled, resulting in high viscosities that depend strongly on molecular weight.⁴ The endohydrolysed CMC product is expected to have a lower molecular weight and less entangling of chains resulting in а decreasing viscosity.

BACKGROUND

Substrate CMC

CMC is a polymer consisting of a number of 1-4- β -linked glucose units. The CMC polymer chain has two ends, which are conventionally referred to as the reducing



Figure 1. Schematic overview of CMC (top) and how exocellulases and endoglucanases, respectively, hydrolyse CMC. Large circles mark ether oxygens or aldehyde end-group oxygen. Exoenzymes (left) result in cellobiose and a new CMC end group.

end, due to an aldehyde group, when in open conformation, and a non-reducing end, respectively.⁵ Additionally, carboxymethylgroups (see Fig. 1) are linked to the OHgroups of the glucose units through an etherbond. Sodium ions bind to the carboxylate group.

Degree of substitution (DS) is the average number of carboxymethyl-groups per glucose unit. The maximum DS is three, so each glucose unit can at most have three carboxymethyl substituted groups.⁴ The carboxymethyl-groups are randomly distributed along the cellulose chain,⁵ which means that some areas of a chain are most likely more substituted than others. The enzyme's ability to hydrolyse is greatly influenced by how substituted the glucose units are. If a chain is highly substituted it will be less hydrolysed by enzymes.⁶ CMC substrates are available with different DS and different degrees of polymerization (DP). DP and DS affect the size of the molecule, the viscosity, and the solubility of CMC. The side chains of CMC make it highly hydrophilic. The longer the polymer chain of CMC, the less soluble is CMC.

Enzymes

Cellulases adsorb to cellulose substrates and catalyze the hydrolysis of the $1,4-\beta$ -Dglycosidic bonds. Exocellulases hydrolyse cellulose from either the reducing or the non-reducing end, whereas endoglucanases the chain randomly.⁵ hydrolyse The hydrolysis product from exocellulases is mostly cellobiose (Fig. 1), with some glucose and cellotriose.⁵ Cel6a and Cel7a from Trichoderma both reesi are characterized as processive exocellulases. They both consist of three structural parts: a catalytic domain (CD), a linker and a cellulose-binding module (CBM).⁷ The differences in the CD of the enzymes and their tunnels, give the enzymes different cleaving capabilities. Typically closed tunnels and rigid CDs favor a processive exo-manner of hydrolyzing whereas a more open tunnel and flexible CD structure favors an endo manner.⁵ Endoglucanase 3 (EGIII) is a pure endo-enzyme and does not have a CBM. The CD of EGIII does not contain a tunnel but a cleft/crevice that is more open than a tunnel, thus enabling its pure endo properties.⁸ Cel6a is thought to possess more endo-character than Cel7a, due to the

more open tunnel and flexible CD, but this is much debated and unclear at present.^{9,10}

MATERIALS AND METHODS Substrate Solutions

CMC (DS = 0.90) with average molecular weight (M_w) of 90 kDa and Avicel ph-101 (regenerated cellulose) were from Sigma (St. Louis, MO) and used without purification. A 50mM acetate buffer with a pH of 5.00 was used in all experiments.

Enzymes

Three different wild type enzymes Cel6a, Cel7a and EGIII from *Trichoderma reesei* were kindly provided by Novozymes at stock concentrations of 77, 173 and 327 μ M, respectively.

PAHBAH Reducing Sugar Assay

A p-hydroxybenzoic acid hydrazide (PAHBAH) assay was performed according to the method described in Lever¹¹ with 400 μ L 50 g/L Avicel as substrate and 50 μ L enzyme solutions at different concentrations to investigate the amount of hydrolysis in 60 minutes. The results reflect the number of reducing ends produced on soluble oligosaccharides and were used to obtain the concentrations corresponding to the same enzyme activity of the three enzymes.

Viscometry

Steady shear viscosities were measured by use of a Bohlin VOR rheometer with a C14 couette geometry. Shear rate sweeps between 1 and 1000 s⁻¹ at 25 °C showed that solutions were virtually Newtonian except at the highest concentrations and rates. All experiments were done in the Newtonian range typically between 10 and 100 s⁻¹. To follow the effect of enzymes with same activities 2.4 mL 5 wt% CMC solutions were first transferred to the measuring cell and measured for about 10 minutes at the chosen shear rate. 2-20 μ L enzyme solution were then added directly to the CMC solution in the cell depending on the concentration and activity of the stock enzyme solutions. Solutions were then mixed quickly in the cell and viscosities measured as a function of time for 40-60 minutes at the chosen shear rate.

An Ubbelohde capillary viscometer from Schott-Geräde was used to determine intrinsic viscosities at 25.0°C. Buffer flow time was about 80 s and a series of CMC concentrations were measured. The intrinsic viscosity was determined from extrapolation of specific and inherent viscosities to vanishing concentrations.

Size Exclusion Chromatography

A Superose HR6 column (GE Healthcare) (25 cm and 4.4 mm diameter) at a flow rate of 0.2 mL/min and a Knauer HPLC system with RI-detector. The acetate buffer was used as eluent. 50 μ L 0.1 wt% CMC solutions were injected on the column.



Figure 2. Specific (circles) and inherent (squares) viscosities of CMC 90 kDa solutions. Intrinsic viscosity is obtained from the intercept at vanishing concentrations.

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RESULTS

Intrinsic viscosity is a measure of the specific hydrodynamic volume of the polymer. The determination of the intrinsic viscosity for the 90kDa CMC used in this study is shown in Fig. 2.



Figure 3. Steady shear viscosities of CMC 90 kDa solutions from capillary (filled circles) and rheometer (squares) measurements.

The intrinsic viscosity $[\eta]$ is 183 ± 2 mL/g. The overlap concentration which marks the change from dilute to semi-dilute solutions is thus c* $\approx 1/[\eta] = 0.0055$ g/mL.

Fig. 3 shows the viscosities as a function of concentration of CMC from dilute to semi-dilute solutions. In the dilute area, the viscosity is slowly increasing with concentration, however, above the overlap concentration the viscosity increases dramatically in the semi-dilute range.

In this study the viscosity should be in the semi-dilute range due to the fact that the viscosity depends strongly on molecular weight in this area. After the addition of enzyme, the viscosity is expected to decrease dramatically, which makes it important to have a high viscosity to start with. For this study a 5% solution was chosen and the overlap parameter $c[\eta]$ of this solution is 9.2. The solutions are thus semi-dilute and measured viscosities are 180 mPas at 25 °C.



Figure 4. Time dependent steady shear viscosity of 5 wt% CMC after addition of EGIII to 0.05 µM at 25 °C. Relative viscosity is measured viscosity divided by viscosity of CMC solution before addition of enzyme.



Figure 5. Relative viscosity of 5 wt% CMC solution after addition of EGIII. (A): 0.05 μM, 25 °C; (B): 0.50 μM, 25 °C; (C): 0.05 μM, 40 °C.

The three different enzymes at different concentrations were added at time 0. The viscosities are normalized relative to the CMC solution viscosity after a small correction due to the dilution with enzymes. The addition of the endoglucanase EGIII decreased the viscosity dramatically with time (see Fig. 4). Both temperature and enzyme concentration are important, as shown in Fig. 5. As seen from Fig. 5, viscosities decrease faster for EGIII additions at higher temperatures and with higher enzyme concentrations.



Figure 6. Relative viscosity of 5 wt% CMC solution after addition of Cel7a. Conditions: 0.05 and 0.5 μ M at 25 °C and 0.05 μ M at 40



Figure 7. Relative viscosity of 5 wt% CMC solution after addition of Cel6a. Conditions: 0.50 µM at either 25 or 40 °C.

No decrease in viscosity was observed at any conditions for both Cel6a and Cel7a, indicating no endo-hydrolysis for these enzymes, as shown in Figs. 6 and 7.

Figure 8 shows SEC-chromatograms of CMC solutions with either Cel7a or EGIII after 20 minutes of hydrolysis. The untreated CMC solution shows a broad molecular weight distribution near the exclusion limit of the column. The smaller later peaks are close to the total volume of the column and may be small saccharides, salts or impurities in the CMC sample. When CMC solution was injected after 20 min hydrolysis with EGIII, the peaks shift to the right, showing that the CMC has been cut to smaller polymer fragments, indicating endoactivity. Only minor changes were observed when Cel7a was added for the large size components, but it is seen that the possible disaccharide peak increases slightly, indicating that this enzyme is exoactive and is only able to cleave at the ends of CMC polymer chains.



Figure 8. Size exclusion chromatograms (SEC) of CMC solutions before addition of enzyme and after 20 minutes of hydrolysis with EGIII and Cel7a. Internal standard elutes at total column volume of 14 mL.

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DISCUSSION

The results obtained for EGIII and Cel7a demonstrate that EGIII is an endoglucanase and that Cel7a is an exocellulase in agreement with other studies.^{5,7,8} EGIII showed measureable endo-hydrolysis, which was observed with both viscometry and SEC, whereas no endo-hydrolysis was observed for Cel7a. Cel6a is generally characterized as an exocellulase, however it has been hypothesized that Cel6a also possess some endo-activity.^{5,10,12} In this study, no endo-activity was observed for Cel6a. A possible explanation for Cel6a showing endo-activity is the choice of substrate. Boisset et al.¹⁰ used bacterial cellulose which is a highly crystalline and insoluble form of cellulose. Cel6a might be more specific for this substrate than for CMC. The catalytic tunnel of Cel6a contains six substrate sites, four for substrate (four linked glucose units) and two for product (one cellobiose unit). The tunnel is very narrow, making almost no space for substituted side-groups. This means that Cel6a has to bind to at least 4 unsubstituted glucose units to be able to hydrolyse. However, the CMC substrate used in this study has a DS of 0.85, which makes is highly unlikely that 4 unsubstituted glucose units are located together. Cel7a has six substrate binding sites, and EGIII has only two, which also reflects the different enzymes ability to hydrolyze CMC.^{5,8}

The DS of CMC affects the hydrolysis. In a study by Horner et al.⁶ it was shown that hydrolysis by EGIII was more effective when the CMC substrate had a DS of 0.6 compared to a DS of 1.2. Using HPLC, it was detected that CMC 0.6 had been hydrolyzed more than CMC 1.2. Cel6a substrate specificity was also illustrated when Avicel was used as substrate in PAHBAH assays. Cel6a released more than twice the product amount compared to the two other enzymes. This could indicate that Cel6a has a higher activity for crystalline cellulose. Boisset et al. investigated Cel6a by transmission electron microscopy with bacterial cellulose as substrate. They detected ribbon shortening when substrate was incubated with Cel6a.¹⁰

Viscometry is clearly a good and sensitive method for detecting endo-activity on CMC substrates. However, insoluble cellulose substrates cannot be measured, which is a significant limitation of the technique. In addition. neither exo-activity nor processivity is detectable with this method. Viscometry is dependent on the MW of the substrate, and only endo-hydrolysis changes the MW significantly. This is due to the relationship between MW and viscosity. Kulicke et al.⁴ have shown that the viscosities of CMC solutions follow a universal curve independent of DP and DS when plotted against the overlap factor. There results show that the viscosity is proportional to MW^{3.9} in semi-dilute solutions. If CMC chains on average are cut only once viscosities will decrease by a factor of 1/15.

Viscometry enables monitoring of endohydrolysis in real-time making it possible to instantly see if the enzymes possess endo activity, without further chemical treatment or analysis.

CONCLUSION

In this study it can be concluded that EGIII endo-hydrolysis can be detected with viscometry. The results from viscometry correlated with the results from both the SEC and PAHBAH assays. Viscometry is a rapid, reliable and sensitive method to identify pure endoglucanases in real time. In addition, both the viscometry and SEC results showed that Cel6a has no detectable endo-activity when CMC was used as substrate.

Further experiments with other endoglucanases are desirable to support the results of this article and to validate that viscometry is a good and fast method to screen for endo-hydrolysis in general.

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