Partial purification of endo-1,4-β-D-glucanase I of *Trichoderma reesei* and use in small-scale enzymatic hydrolysis of cellulose

By

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Abstract

In order to reduce the cost of lignocellulosic ethanol production, a model was previously developed to predict the amount of fermentable sugar released based on parameters concerning both biomass and enzymes. To validate this model, enzymatic hydrolysis must be carried out using individual purified cellulase enzymes. Endo-1,4-β-D-glucanase I (EG I), a typically difficult enzyme to purify, was partially purified using three steps of fast protein liquid chromatography. The purity and identity were determined using SDS-PAGE and specific activity assays. The partially purified fractions of EG I, and three other purified cellulase enzymes were used to hydrolyze three different cellulose types. The three enzyme mixtures, with increasing fractions of EG I, showed insignificant differences for all three types of cellulose used.

Keywords: biofuels, lignocellulosic ethanol, protein purification, cellulase, enzymatic hydrolysis

1. Introduction

As global warming continues to be a major concern, mass efforts have been made to reduce the net CO_2 emissions caused by the transportation sector. One proposed solution is to develop lignocellulosic ethanol as an alternative fuel source. Despite having well-understood methods of production, the high cost of the end product continues to be a major hurdle to overcome before widespread use of lignocellulosic ethanol becomes a reality.

Lignocellulosic ethanol production is comprised of three major steps; a) biomass pretreatment, b) enzymatic hydrolysis, and c) fermentation. Enzymatic hydrolysis has

been shown to be one of the most expensive steps due to the need for high concentrations of cellulase enzymes to sufficiently hydrolyze biomass (Kumar, 2014; Liu et al., 2017; Merino and Cherry, 2007).

There are three main families of cellulase enzymes which work synergistically to efficiently hydrolyze cellulose. Endoglucanases (EG), which randomly cleave internal bonds of amorphous cellulose chains. This action provides access to the ends of cellulose chains for cellobiohydrolases. Cellobiohydrolases work processively to release cellobiose from either end of a cellulose chain. Specifically, cellobiohydrolase I (CBH I) acts on the reducing end of the cellulose chain, and cellobiohydrolase II (CBH II) acts on the non-reducing end. β-glucosidase (BG) completes the final step by hydrolyzing cellobiose to release fermentable glucose. It should be noted that there are other minor enzymes that have also been identified aiding hydrolysis. These enzymes work to modify the cellulose chains to provide better access to the cellulose chain bonds for the main hydrolyzing enzymes (Berlin et al., 2007; Bischof et al., 2016).

Industrial mixtures of cellulase enzymes used for biofuel production are commonly derived from the filamentous fungi, *Trichoderma reesei* (*T. reesei*), this species is an anamorph of *Hypocrea jecorina* (Seidl et al., 2008). Over the years, this fungus has been mutated to overproduce cellulolytic enzyme, reaching up to 30g/L of protein (Bischof et al., 2016). In the presence of cellulose, *T. reesei* excretes an approximate ratio of 40-60% CBH I, 12-20% CBH II, and 5-10% EG I-V (Meyer et al., 2009). Yet studies show this ratio of cellulase enzymes is not optimal for any given type of biomass (Andersen et

al., 2008; Rosgaard et al., 2007). The efficiency of these cellulase enzymes greatly depends on both the parameters of biomass and cellulase enzymes. By optimizing the composition of cellulase enzyme mixtures used for each source of biomass, a smaller volume of enzymes would be needed, leading to a lower cost of production (Bansal, 2009; Meyer et al., 2009).

The current standard for determining optimal enzymatic composition requires running large quantities of small-scale enzymatic hydrolysis experiments. However, these types of experiments can be time-consuming and costly. One proposed solution is to develop a mechanistic model that can approximate the amount of fermentable sugar produced based on multiple parameters of both biomass and enzymes (Bansal, 2009; Kumar and Murthy, 2013). Compared to performing large numbers of hydrolysis experiments, a reasonably accurate model could be a quick solution to determine optimized enzymatic hydrolysis conditions. To determine the validity of the model, controlled enzymatic hydrolysis experiments using purified individual cellulase enzymes are needed.

Obtaining a complete profile of purified cellulase enzymes can be difficult, and in this context most previous work has been done using only 2-4 purified enzymes. (Gama et al., 1998; Medve et al., 1998; Reese, 2013). While methods to purify CBH I and CBH II are well understood, purifying endoglucanases, specifically EG I, presents more difficulties. This is due to EG I having a similar amino acid sequence and size compared to CBH I, as well as a low concentration within a crude mixture (Meyer et al., 2009; Penttilä et al.,

1986). Many studies choose to produce EG I in other organisms to avoid these difficulties. This study used methods previously described by Jaeger et al. to obtain partially purified EG I from Celluclast 1.5 L (Novozymes, Denmark) using three steps of fast protein liquid chromatography (FPLC). Purified CBH I, CBH II, BG, and partially purified EG I were used to perform small-scale hydrolysis experiments to determine effects of designed enzyme compositions on three types of cellulose.

2. Methods

2.1 Protein Purification

Celluclast 1.5 L (Novozymes, Denmark) was used as the source of cellulase enzymes. All steps of FPLC were performed using the BioLogic LP and BioFraction system (Bio-Rad, USA) which measured conductivity and ultraviolet light absorbance at 280 ηm. All chromatography resins were purchased from Sigma-Aldrich, USA. All experiments were performed at room temperature and collected samples were stored in 4°C in between steps.

Celluclast 1.5 L was desalted and rebuffered using a Sephadex G-25 Fine gel filtration column (2.5 cm x 10 cm). The loading buffer used was 50 mM Tris-HCl (pH 7.0), at 5 mL/min. Anion-exchange chromatography was performed using a DEAE Sepharose column (2.5 cm x 10 cm). The sample was loaded using 50 mM Tris-HCl (pH 7.0), at 20 mL/min. Protein was eluted stepwise (35% v/v, 100% v/v) using 50 mM Tris-HCl, 0.2 M NaCl (pH 7.0). The first elution was concentrated and rebuffered in 50 mM sodium

acetate (pH 3.5) using Pellicon XL 50 Ultrafiltration Cassette, with biomax 10 (Millipore, USA). Dilute HCl was added to adjust the sample to a final pH of 3.5. Cation-exchange chromatography was performed using a SP Sepharose Fast Flow column (1.5 cm x 7.5 cm). The sample was loaded using 50 mM sodium acetate (pH 3.5) at 2 mL/min (Jäger et al., 2010). Protein was eluted stepwise (15% v/v, 100% v/v) using 50 mM sodium acetate, 1.0 M NaCl (pH 3.5). CBH I and CBH II were purified using methods previously described (Kumar, 2014) and Novozyme 188 (Sigma-Aldrich, USA) was used as a source of BG.

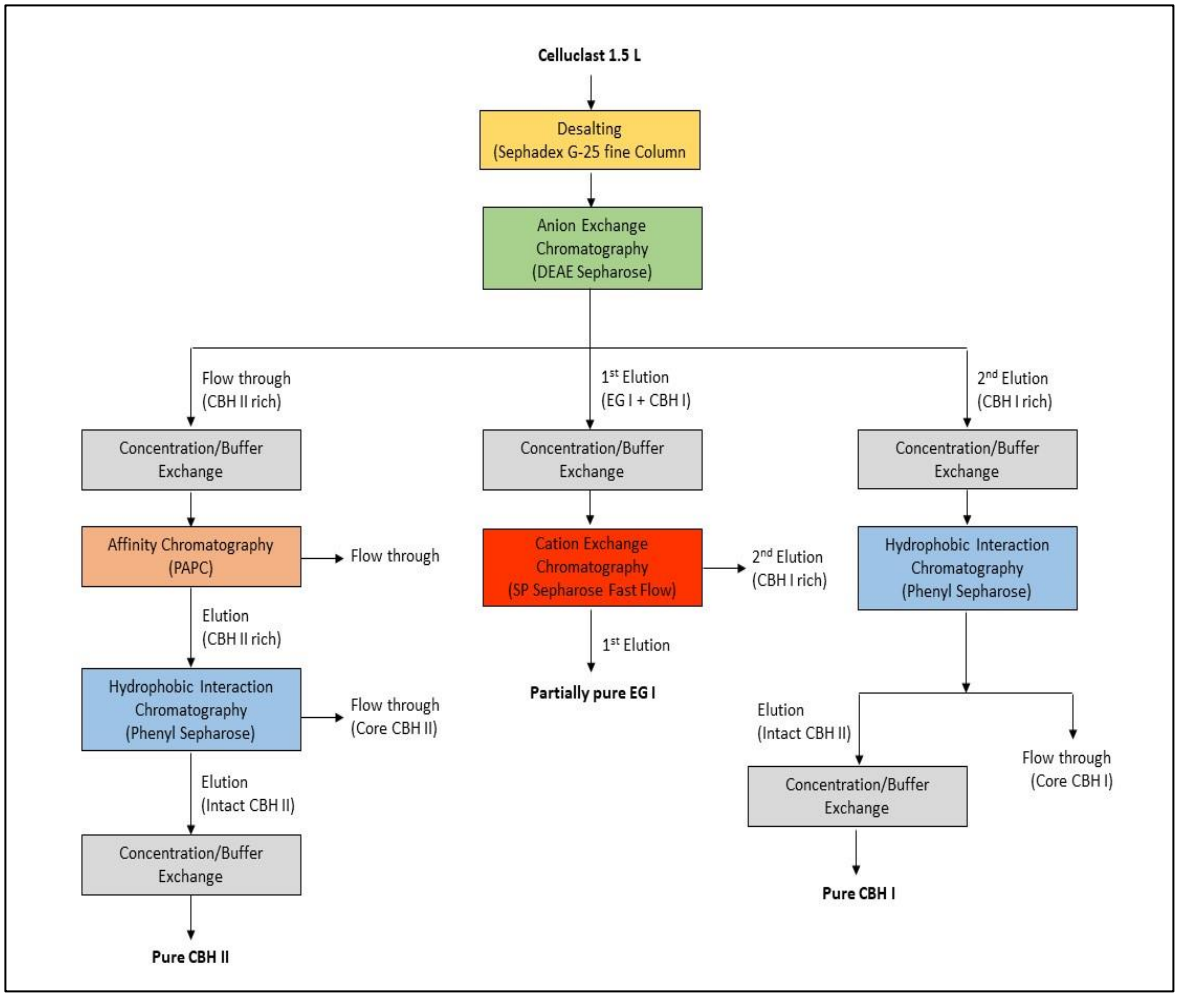

Figure 1. Flow chart of FPLC methods used to purify CBH I and CBH II, and partially purify EG I from Celluclast 1.5 L.

2.2 Measurement of Protein Concentration

The final concentrations of the protein samples were measured using Quick Start™ Bradford Protein Assay (Bio-Rad, USA). The assay was performed in a 96-well plate, using standard protocol ("Quick Start™ Bradford Protein Assay Instruction Manual," n.d.). Bovine serum albumin (Bio-Rad, USA) was used as a standard. Samples were measured 595 nm in a SpectraMax Plus (Molecular Devices, USA).

2.3 Measurement of protein purity

The purity and identity of the samples were determined using SDS-PAGE. Samples were prepared by using 1 mL of 100% trichloroacetic acid (TCA) to precipitate protein from solution. The samples were then centrifuged at 15,000 rpm for 10 minutes. The TCA was removed and the protein pellets were rinsed with 1 mL of chilled acetone. Samples were then centrifuged at 15,000 rpm for 2 minutes. The acetone was removed. 100 µL of 2x laemmli buffer (Bio-Rad, USA), reduced with 5% beta-mercaptoethanol, was added. Samples were heated at 95°C for 5 minutes, and then centrifuged at 15,000 rpm for 2 minutes.

The experiments were performed using 4–20% Mini-PROTEAN TGX Precast Protein Gels, 12-well, 20 µL (Bio-Rad, USA) and 1x Tris/Glycine/SDS was used as running buffer. The gel was run at 87 volts until the dye front reached the bottom of the gel. Precision Plus Protein Standards, unstained (Bio-Rad, USA) were used as a molecular weight marker.

The gel was rinsed in a solution of 10% acetic acid, and 50% methanol overnight. The gel was stained for 4 hours in 10% acetic acid, 50% methanol, and 0.25% Coomassie Blue R-250. The gel was destained overnight in 5% methanol, and 7.5% acetic acid. The gel was fixed in 7% acetic acid.

The sizes of sampled proteins were estimated by calculating the R_f value. The distance of protein bands in the gel were measured manually using a ruler.

2.4 Enzyme Specific Activity

The specific activity of EG I, CBH I, and CBH II were measured to determine purity of the samples. All experiments were performed in a 96-well PCR plate (Xiao et al., 2005). 30 µL of enzyme sample and 30 µL of 2% w/v cellulose in 50 mM sodium acetate (pH 4.8) were mixed in each well. All three protein samples specific activity were measured using Avicel (Sigma-Aldrich, USA) as a cellulose source. Additionally, EG I was measured on carboxymethyl cellulose (Sigma-Aldrich, USA). A solution of 2% w/v D-glucose in 50 mM sodium acetate (pH 4.8) was used as a standard. All samples were performed in replicates of three.

The samples were incubated at 50°C and shaken for 120 minutes. 60 µL of 3,5-dinitrosalicylic acid reagent was added to each sample. The samples were heated at 95°C for 5 minutes, covered with a mat to prevent evaporation of sample. Immediately after, the samples were placed in an ice bath for 5 minutes to quench the reactions. 100 µL of each sample was transferred to a flat bottomed, 96-well plate and measured at 540 ηm in a SpectraMax Plus (Molecular Devices, USA).

2.5 Enzymatic Hydrolysis

Enzymatic hydrolysis was carried out in a 96-well plate. Solutions of 10% w/v cellulose were made using 50 mM sodium acetate buffer (pH 4.8). Three biomass samples were used; carboxymethyl cellulose, Avicel, and bacterial cellulose (Simonsen Lab, Oregon State University). To create bacterial cellulose samples of 10% w/v, the sheets of

cellulose were manually shredded using scissors, individual samples were measured, and then directly submerged into the buffer in the sample wells.

The enzymes were loaded at a total protein concentration of 20 mg/g glucan, which includes 5 mg/g glucan of BG for all treatments. Three treatments were applied to the three types of cellulose.

The remaining protein content, 15 mg/g glucan, was divided between CBHI, CBH II, and EG I in enzyme ratios (CBH I: CBH II: EG I) of 70:25:5, 70:20:10, and 60:20:20 respectively. All treatments were performed in four replicates.

Samples were incubated at 50°C, and constantly shaken for 72 hours. The plate was inverted by hand every hour for 48 hours, then every 2 hours, to keep insolubilized biomass homogenous in solution. After 72 hours the samples were stored in the freezer at -20°C.

A DNS assay was used to measure the amount of fermentable sugars in solution. In a 96-well PCR plate, 50 µL of DNS reagent was combined with 5 µL of sample and 45 µL of 50 mM sodium acetate (pH 4.8) buffer. The plate was covered with a mat, and heated at 95°C for 10 minutes, and then put in an ice bath for 5 minutes. The samples were then measured at 540 ηm. D-glucose was used as a standard.

3. Results and Discussion

3.1 Protein Purification

3.1.1 Measurement of protein purity

The first elution from the cation exchange column, containing EG I, was collected and multiple samples of varying amounts were loaded onto a SDS-PAGE gel. In Figure 1, the EG I sample displayed multiple protein bands and the non-distinct appearance of the largest band suggested low purity of EG I sample. The largest band in the EG I sample, assumed to contain EG I, was estimated with an average result of 66 kDa, which is larger compared to literature (Jäger et al., 2010).

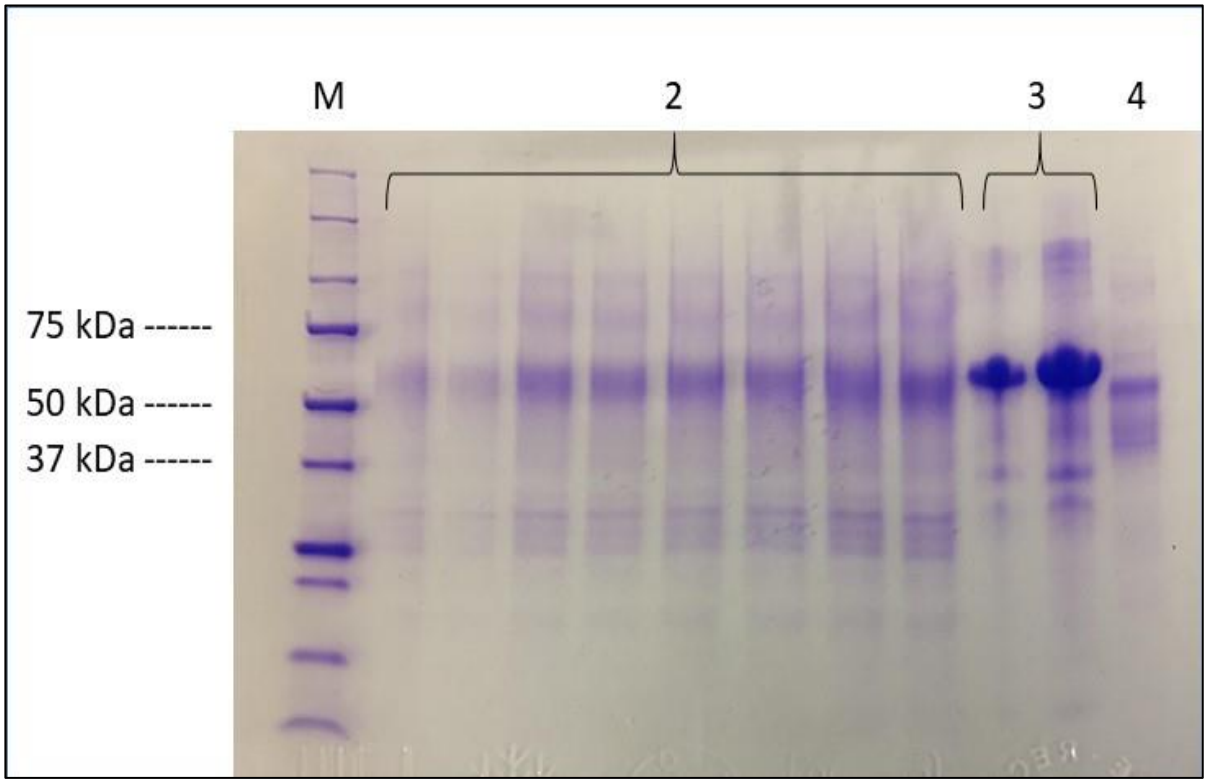

Figure 1. SDS-PAGE gel of (M) molecular marker, (2) EG I sample, (3) purified CBH I, (4) 1st elution from anion exchange column.

3.1.3 Enzyme Specific Activity

The specific activity of the EG I sample and the purified CBH I were tested on Avicel and CMC. All values were an average of 3 replicates. The EG I sample had a specific activity of 0.11 U/mg protein on Avicel. Since the specific activity of the EG I sample on Avicel was higher when compared to the purified CBH I, 0.05 U/mg of protein, it suggested low purity of the EG I sample. The EG I sample had a specific activity of 0.32 U/mg protein on CMC, which suggests there is EG I in the collected sample.

3.2 Enzymatic Hydrolysis

Enzymatic hydrolysis experiments were performed using partially purified EG I. As seen in Table 1, there were no significant difference recorded between treatments for any time of cellulose used. P-values were calculated using the one-way ANOVA method. The results showed that the structure of biomass had a greater effect on the yield of glucose compared to the difference caused by enzyme treatments. One explanation could be the high concentration of enzyme. At 20 mg/g glucan, even the lowest fraction of EG I used could have been sufficient to eliminate the rate limiting effect caused by EG I during hydrolysis of cellulose. Therefore, increasing amounts of EG I may have been unnecessary. Furthermore, the jamming effect created at high concentrations of enzyme may reduce the overall impact of synergistic enzymatic activity. Performing experiments with lower concentrations of enzymes may show more significant differences between treatments.

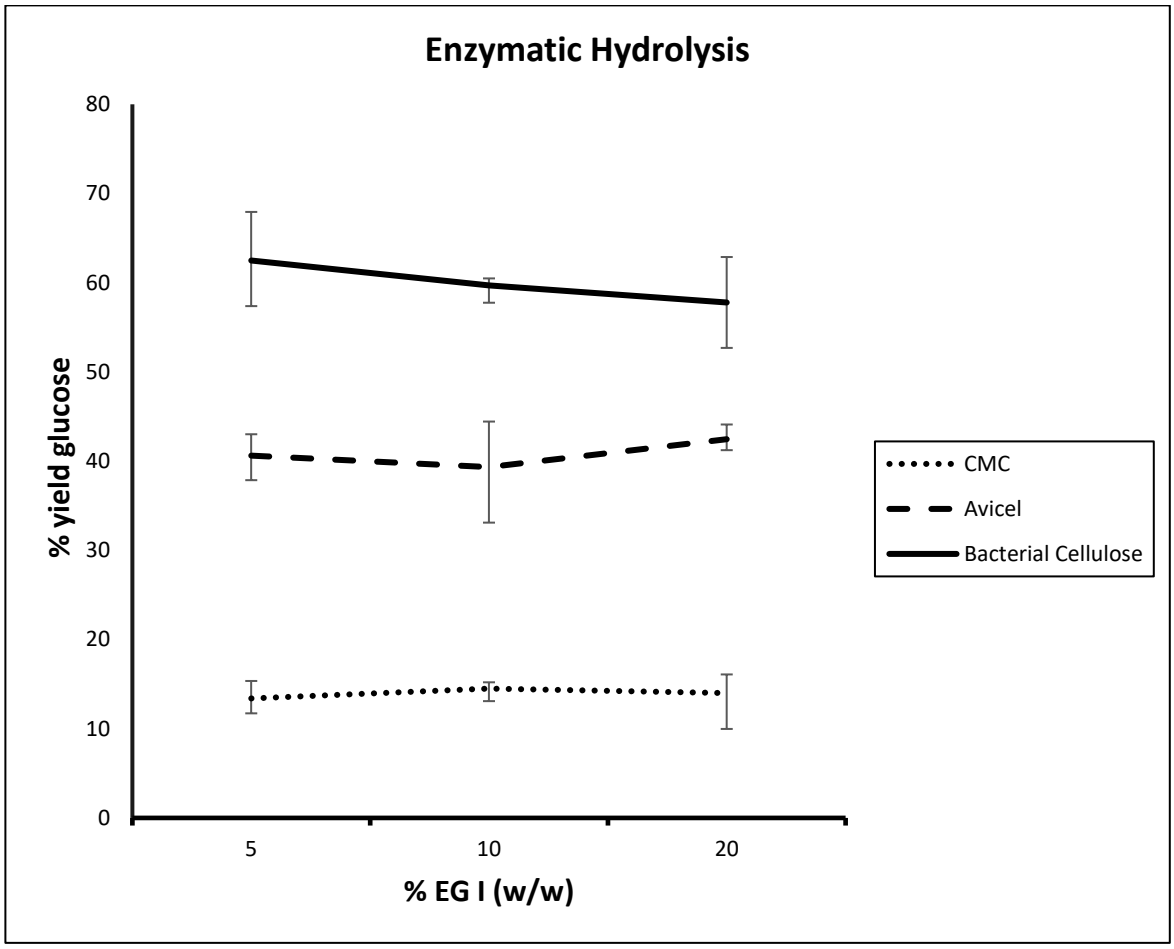

Figure 2. % yield of glucose for all three enzymatic hydrolysis treatments, and biomasses based on increasing fraction of EG I sample in the enzyme mixture (data points are the mean of four replicates).

	% Yield of glucose		
	CMC	**Avicel**	**Bacterial Cellulose**
Treatment 1	13.4	40.6	62.5
Treatment 2	14.5	39.3	59.7
Treatment 3	14.0	42.5	57.8
p-value	0.7	0.4	0.3

Table 1. % yield of glucose for all three enzymatic hydrolysis treatments, with calculated p-values for each biomass type (data represented is the mean of four replicates).

4. Conclusion

In summary, the FPLC methods used were only able to partially purify EG I from Celluclast. This can be concluded from the results of SDS-PAGE, where the EG I sample displayed a large indistinct band with a larger kDa value compared to literature. Also, the high specific activity of the EG I sample on Avicel suggested low purity. No significant difference of the treatments using the partially purified sample of EG I, in increasing amount, was recorded with three types of cellulose which could have been due to the over saturation of enzyme. Lower concentrations of enzyme may bring out the rate limiting effects of EG I during the hydrolysis of cellulose not seen in these results.

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