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Determination of Product Inhibition of CBH1, CBH2, and EG1 Using a Novel Cellulase Activity Assay

Faye Du · Erin Wolger · Louise Wallace · Amy Liu · Thijs Kaper · Brad Kelemen

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Abstract The hydrolysis of lignocellulosic biomass by degrading enzymes (cellulases) has emerged as a promising process within the bio-ethanol industry. Yet, understanding all the intricacies of how these enzymes work has been a challenging task. Substrate–enzyme interaction in complex feed mixtures, the recalcitrance of the crystalline structure of cellulose and enzyme inactivation by product inhibition, nonproductive binding to lignin, and process stress are only some of the problems standing in the way of creating an effective and efficient process to bio-ethanol production. This study focuses on the product inhibition of cellobiohydrolases and endoglucanases. Here, we present a method of studying product inhibition by measuring the decrease in substrate, utilizing the fluorescent properties of a calcofluor dye.

Keywords Calcofluor cellulase assay · Product inhibition

Introduction

There are three groups of enzymes (cellobiohydrolases, endoglucanases, and β-glucosidases) found in cellulases that work synergistically to degrade cellulose. It is known that all three groups can be inhibited by intermediate (cellobiose) or final (glucose) hydrolysis products. Because product inhibition stands out as one of the major limiting factors in lignocellulosic biomass hydrolysis, we are interested in engineering our cellulases to resist product inhibition. The direct measurement of product is difficult in the presence of large amounts of product. Instead, we developed a method of measuring the decrease in substrate taking advantage of the fluorescent properties of a calcofluor dye. Calcofluor white is a nonspecific fluorochrome that binds to cellulose and exhibits a greater fluorescence when in the presence of intact cellulose (Fig. 1). It is routinely used in the textile industry as an optical brightener of white cotton fabrics and in microbiology as an

F. Du (✉) · E. Wolger · L. Wallace · A. Liu · T. Kaper · B. Kelemen
Genencor, a Danisco Division, 925 Page Mill Road, Palo Alto 94304 CA, USA
e-mail: faye.du@danisco.com

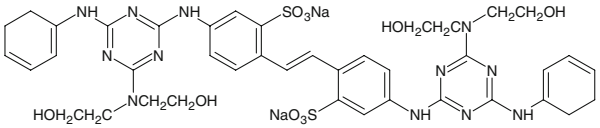

Fig. 1 Chemical structure of calcofluor white [6]

indicator of beta-glucan containing cell walls. We wondered whether the fluorescent dye could be used for quantitative detection of cellulase activity assay as well. Specifically, we looked at the hydrolysis of phosphoric acid swollen cellulose (PASC) by cellobiohydrolases and endoglucanases by detecting the decrease in fluorescence of the calcofluor. We found that while cellobiohydrolase 1 (CBH1) is strongly inhibited by cellobiose, cellobiohydrolase 2 (CBH2) and endoglucanase 1 (EG1) are not as significantly inhibited.

Materials and Methods

Chemicals

All chemicals used were of analytical grade. Avicel PH-101 was purchased from FMC BioPolymer (Philadelphia, PA, USA). Cellobiose and calcofluor white were purchased from Sigma (St. Louise, MO, USA).

Preparation of PASC

Phosphoric acid swollen cellulose was prepared from Avicel PH-101 using an adapted protocol of Walseth and Wood [1, 2]. In short, Avicel is solubilized in concentrated phosphoric acid then precipitated using cold deionized water. After the cellulose is collected and washed with more water to neutralize the pH, it is diluted to 1% solids in 50 mM sodium acetate, pH 5.

Purification of CBH1, CBH2, and EG1

CBH1, CBH2, and EG1 were produced by *Trichoderma reesei* fermentation following the protocol described by Foreman et al. [3]. Essentially, CBH1 was desalted using a Sephadex G-25 column (GE Healthcare, New Jersey, CA, USA). Collected protein was diluted 20 times in MilliQ water and loaded onto a high-density quaternary amine column, POROS HQ (Applied Biosystems, Foster City, CA, USA) equilibrated with 25 mM *N*-tris (hydroxymethyl)-2-aminoethanesulfonic acid (TES), pH 6.8. Protein was eluted with 250 mM TES, pH 6.8, containing 100 mM ammonium acetate. Fractions containing pure CBH1 were combined. CBH2 was purified to 95% purity using a HiLoad 26/60 Superdex gel filtration column (GE Healthcare) equilibrated with 25 mM TES, pH 6.8. The protein was eluted using 25 mM TES, pH 6.8, containing 100 mM NaCl. Fractions containing purified CBH2 were collected and combined. For purification of EG1, *T.* reesei fermentation broth was concentrated using ultrafiltration then loaded on to a hydrophobic interaction POROS Phenyl column (Applied Biosystems, Foster City, CA, USA) equilibrated with 20 mM sodium phosphate, pH 6.8. Protein was eluted with 20 mM sodium phosphate, pH 6.8, containing 0.3 M ammonium sulfate, and fractions containing

EG1 were combined. The CBH1, CBH2, and EG1 preparations used in this study were more than 98%, 98%, and 85% pure as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis, respectively.

Cellulase Activity Assay Using Calcofluor White

Enzymatic activity was tested for hydrolysis of phosphoric acid swollen cellulose at 50 °C for 2 h in the presence of cellobiose. Purified CBH1 and CBH2 were serially diluted to 0, 0.125, 0.250, 0.50, and 1 mg/ml in 50 mM sodium acetate, pH 5. EG1 was serially diluted in 50 mM sodium acetate, pH 5, to 0, 0.098, 0.195, and 0.39 mg/ml. Similarly, cellobiose was diluted to 0, 0.25, 0.50, 1.0, and 2.0 mg/ml. Phosphoric acid swollen cellulose was diluted with 50 mM sodium acetate, pH 5, to 0.25% cellulose. The calcofluor quench was prepared by adding calcofluor white to a final concentration of 50 μg/ml of 100 mM glycine, pH 10.

Each enzyme dose was tested at each concentration of cellobiose. In a microtiter plate, 10 μl of enzyme was added to a well containing 50 μl 0.25% PASC and 50 μl cellobiose. Wells containing buffer and cellobiose only and buffer only were also included on the plate as baseline fluorescence and negative controls. After a minute of vigorous shaking, the plate was placed into an incubator at 50 °C for 2 h. At the end of which, the reaction was quenched with 100 μl of the calcofluor quench buffer. Fluorescence was recorded using a fluorescence plate reader (SpectraMax M5 by Molecular Devices) at excitation wavelength of 365 nm and an emission wavelength of 435 nm.

Due to the unique ability of the calcofluor to bind cellulose, any PASC remaining in the well will bind to the calcofluor and fluoresce. Thus, a high fluorescence output corresponds to a large amount of substrate and, therefore, low enzymatic activity. Conversely, a low fluorescence output means little substrate remaining and, thus, high enzymatic activity. Here, we express the result as the fraction product determined using the equation,

$$F_{\mathrm{P}} = 1 - \left(\mathrm{Fl}_{\mathrm{sample}} - \mathrm{Fl}_{\mathrm{buffer+cellobiose}}\right) / \left(\mathrm{Fl}_{\mathrm{zero\ enzyme}} - \mathrm{Fl}_{\mathrm{buffer+cellobiose}}\right),$$

where F_{P} is the fraction product and Fl=fluorescence units.

p-Hydroxybenzoic Acid Hydrazide Reducing Sugar Assay

A modified method of the *p*-hydroxybenzoic acid hydrazide (PAHBAH) assay [4] was used to analyze samples run in parallel to this experiment to compare the results between

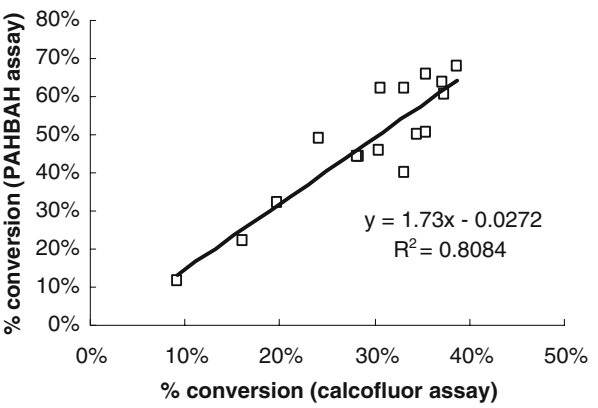

Fig. 2 Correlation between the calcofluor assay and PAHBAH reducing sugar assay

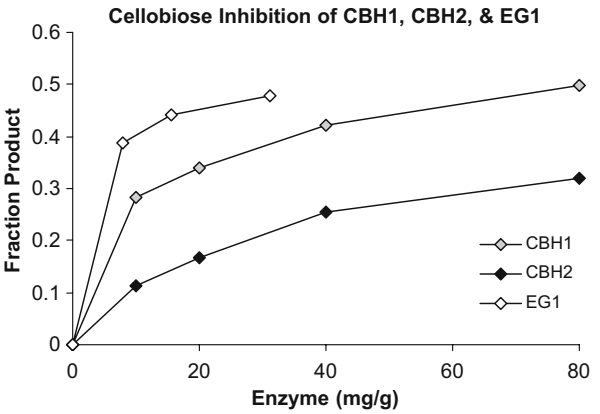

Fig. 3 Cellulase activity of CBH1, CBH2, and EG1 in 0.125% PASC after 2-h incubation at 50 °C. Activity measured by fluorescence of calcofluor white bound to remaining cellulose in reaction mix

the two assays. Data were processed to calculate the percent conversion determined by the equation:

$$\%\ \text{conv} = (\text{well} - \text{background}) \times 100\%/(\text{no enzyme control} - \text{background}).$$

Results and Discussion

The activity assay was first developed from the idea that we could use the cellulose binding properties of the calcofluor white to measure cellulase activity. From literature, we knew that the fluorochrome had binding specificity for hexopyranose polymers with β-configuration like cellulose, carboxymethylcellulose, diethylaminoethylcellulose, and chitin [5]. Initial experiments narrowed the optimal range of calcofluor in solution to 50–200 μg/ml with correlation to the PAHBAH assay with R^2 value of 0.80–0.93 (Fig. 2).

The application of the cellulase activity assay using calcofluor white provided a fast method of screening enzymes for product inhibition. Here, we utilize this method to determine the product inhibition of CBH1, CBH2, and EG1. When no cellobiose is added to the reaction, the relative activities of the three enzymes can be compared (Fig. 3).

When cellobiose is added in increasing concentrations, we observed minimal inhibition of the CBH2 molecule while CBH1's activity is significantly affected by the cellobiose. In our assay, we conclude that CBH1 is strongly inhibited by cellobiose while CBH2 and EG1 display less inhibition by this intermediate product (Fig. 4).

The cellulase activity assay using calcofluor white has proven to be an effective tool in measuring cellulase activity on a variety of substrates including phosphoric acid swollen

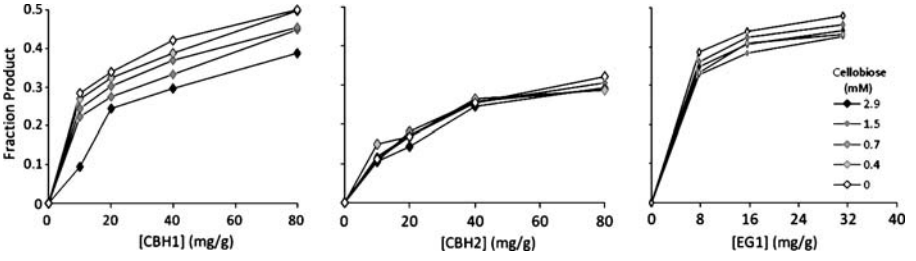

Fig. 4 Activity of CBH1, CBH2, and EG1 on 0.125% PASC in the presence of various concentrations of cellobiose (0–2.9 mM)

cellulose and carboxymethylcellulose. It has provided us with the ability to screen a multitude of enzymes in a fast and efficient manner that can also be applied as a high-throughput assay.

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