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A Simplified Filter Paper Assay Method of Cellulase Enzymes Based on HPLC Analysis

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Abstract A simplified filter paper assay (FPA) method of cellulase enzymes was proposed based on high-performance liquid chromatography (HPLC) measurement. The method was according to the sum of glucose and cellobiose concentrations measured by HPLC that was able to be correlated with filter paper units (FPU) of the cellulase enzymes assayed by the traditional FPA method, regardless of the differences in the sources, activities, and components of the cellulases. This simple and quick assay method for the cellulase enzymes provided another parameter of the ratio of glucose to cellobiose (G/C ratio) representing the capacity of cellulase enzymes degrading cellulose into fermentable monomeric sugars.

Keywords Filter paper activity · Cellulase · Simplified method · HPLC measurement · Corn stover

Introduction

A quick and reliable cellulase activity assay is important for evaluation of the efficacy of cellulase enzymes and the development of new enzymes. Various assay methods had been developed since 1950s, and among these methods, the filter paper assay (FPA) method, was most widely used [1]. The method used Whatman No. 1 filter paper as substrate, and the filter paper was degraded by using a series of diluted cellulase solutions to get a fixed degree of substrate conversion. DNS (3, 5-dinitrosalicylic acid) reagent was used for determination of reducing sugars released by identifying the aldehyde groups at the sugar chain ends. This method had almost become the standard method for evaluating cellulase activities [2] but is very time consuming [3, 4]. This method was modified to reduce assay errors and facilitate

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high-throughput assay [5–7], but a simple method is still required for improving the accuracy and efficiency of cellulase enzyme assay.

On the other hand, commercial cellulases are mostly a mixture of enzymes containing endoglucanases, exoglucanases, and β-glucosidases. Endoglucanases can just split cellulose chains while sugars hardly formed. The synergic behavior of these enzymes is crucially important for obtaining utilizable sugars. Therefore, a method of evaluating the cellulase for converting cellulose to available sugars is of great interest. For cellulase derived from *Trichoderma reesei*, the sufficient supplement of β-glucosidase to the cellulase enzymes is required. The supplement of β-glucosidase also improves the filter paper unit (FPU) of the cellulase, but it makes no difference among the enhancement of different cellulase components in the current FPA assay. Thus, the present FPA method is not suitable for searching the optimal synergy of the cellulase complex.

Currently, the sugar analysis using high-performance liquid chromatography (HPLC) is the generally practiced method for determination of lignocellulose-derived sugars such as glucose and cellobiose. HPLC was compatible with the DNS method for determining sugars. Moreover, HPLC was a more in-depth analytical method compared with the DNS method. Fox et al. [8] found that a discrepancy existed between sugar analysis using HPLC and DNS methods because HPLC was specific for determining the concentration of glucose, xylose, and cellobiose while DNS could react with any substances of reducing ends. Rivers et al. [9] showed that the DNS method was susceptible to interferences and methods like HPLC should be used for sugar analysis in the enzymatic hydrolysis. In fact, formation of components, such as glucose and cellobiose, representing most of the final products could sufficiently reflect the hydrolysis ability of a cellulase enzyme.

In this study, a simplified FPA method of cellulase enzymes using HPLC measurement was proposed and compared to the traditional FPA method. The same filter paper hydrolysis was carried out for the simplified method but the concentrations of glucose and cellobiose were measured using HPLC, instead of the DNS method for reducing sugar concentration. The sum of glucose and cellobiose concentrations was correlated with the FPU data obtained from the traditional FPA method. The HPLC-based method for FPU assay also yielded a useful parameter in terms of the ratio of glucose to cellobiose (G/C ratio) for evaluation of cellulase synergy. The method developed in this work gave a simple and rapid FPA of cellulase enzymes and might be used as a general method of cellulase activity assay.

Materials and Methods

Enzymes and Materials

The commercial cellulase enzymes used included Accellerase 1000 and Spezyme CP from Genencor International (Rochester, NY, USA); Youtell #5 derived from *T. reesei* was purchased from Hunan Youtell Biochemical Co. (Yueyang, Hunan, China). Whatman No. 1 filter paper was purchased from Whatman International (Maidstone, UK). All other reagent-grade chemicals were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China). Corn stover (CS) was grown in Jilin province, China and harvested in fall, 2007.

Determination of Filter Paper Activity Using the Standard Method

The FPA of cellulase enzymes was carried out in a mixture containing 0.5 mL diluted enzyme by 50 mM citrate buffer (pH 4.8) and 50 mg of Whatman No. 1 filter paper and then

incubated at 50 °C for 1 h as described by Adney and Baker [10] and Ghose [2]. The reducing sugars released were determined using the DNS method. FPU was defined as 0.37 divided by the amount of enzyme that produced 2.0 mg glucose equivalents in 1 h from 50 mg of filter paper. All experiments were carried out in triplicates.

Determination of Filter Paper Activity Based on the HPLC Measurement Method

The same filter paper hydrolysis procedure above for the traditional assay using the DNS method was applied. For sugar concentration measurement, the HPLC method was used for the determination of glucose and cellobiose concentrations, instead of the DNS method for the reducing sugar determination. Each enzyme dilution was diluted 100-fold with citrate buffer, and the hydrolysis of filter paper was carried out in a mixture containing 0.5 mL of the 100-fold diluted enzyme, 1 mL of citrate buffer, and 50 mg of Whatman No. 1 filter paper incubated at 50 °C for 1 h. The hydrolysate was immediately chilled into the ice water and centrifuged at 13,000 rpm for 5 min. Again the supernatant was chilled into the ice water and then used for glucose and cellobiose concentration measurement on HPLC. The sum of glucose and cellobiose concentrations was correlated to the FPU data determined using the traditional FPA method as described above for each enzyme dilution.

Pretreatment and Enzymatic Hydrolysis of Corn Stover (CS)

The dilute sulfuric acid pretreatment of CS was carried out as described by Zhang et al. [11]. The conditions for the dilute acid pretreatment of CS used in this study was at 190 °C for residence time of 3 min using the 2.5-g sulfuric acid per gram of dry mass at the full CS filling of the pretreatment reactor and the solid/liquid presoaking ratio of 2.0. The enzymatic hydrolysis of the pretreated CS was conducted in a 250-mL shake flask incubated with a work volume of 50 mL of 50 mM citrate buffer (pH 4.8) at 10 % (w/w) solids loading at 50 °C and 150 rpm for 24 h. The cellulase dosage was 10 FPU/g dry mass. All experiments were conducted in triplicate.

HPLC Analysis

Glucose and cellobiose were analyzed using an HPLC (LC-20AD, refractive index detector RID-10A, Shimadzu, Kyoto, Japan) equipped with an Bio-Rad Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA) at the column temperature of 65 °C with 5 mM H_2SO_4 as eluent with a flow rate of 0.6 mL/min. Samples from enzymatic hydrolysis were centrifuged at 13,000 rpm for 5 min and then filtered through a 0.22-μm filter before injection.

Results and Discussion

HPLC-Based Assay of Filter Paper Units (FPU) of Cellulase Enzymes

The three commercial cellulases, Accellerase 1000, Spezyme CP, and Youtell #5, were diluted with citrate buffer into four samples: undiluted, twofold, threefold, and fourfold. First, the FPU of the samples were determined by measuring the reducing sugars using the DNS method according to the standard FPU assay procedure. Then, following the procedure of the HPLC measurement method, the filter paper hydrolysis was carried out for 1 h at 50 °C,

and the glucose and cellobiose concentrations of the filter paper hydrolysate was determined on HPLC. Finally, the logarithmic values of the FPU of the three commercial cellulases versus the sugar concentrations measured by the HPLC were drawn in Fig. 1. Figure 1a indicates that the correlation was poor using the glucose concentration alone as against the FPU values determined using the traditional FPA method. However, when the FPU were correlated with the sum of glucose and cellobiose concentrations, an approximately linear semilogarithmic proportion was observed as shown in Fig. 1b. It is noticed that although the cellulase enzymes come from different sources and components in the different dilutions, all the data were falling onto the same line approximately with limited data scattering. The FPU values determined using the standard assay method was correlated well with the sum of glucose and cellobiose. The results indicate that the FPU value of cellulase enzymes can be estimated from the HPLC based the concentrations of glucose and cellobiose of the filter paper hydrolysate. The concentrations of glucose and cellobiose after enzymatic hydrolysis can be measured conveniently using HPLC, have more accurate and less data scattering, and have less time consumed than the DNS method in the current FPU assay procedure.

When the filter paper tested was hydrolyzed, various oligomeric sugars existed inevitably in the filter paper hydrolysate. Practically, it was not easy to measure them all quantitatively using the general HPLC machine. Figure 1b demonstrated that the sum of glucose and cellobiose versus the FPU values of the three commercial cellulase enzymes could give a satisfactory correlation. Therefore, it is reasonable to estimate the FPU value of a cellulase enzyme by correlating the glucose and cellobiose concentrations only, instead of the measurement of the whole spectrum of the sugars.

However, when this correlation of the sum of glucose and cellobiose concentrations to the FPU value of a specific cellulase enzyme is applied, it is recommended to establish a specific correlation equation experimentally for the specific enzyme, instead of using the listed correlation in Fig. 1b to assure sufficient accuracy. Except for the measurement of the commercial cellulase, the HPLC-based method could be applicable to the assay of the cellulase with low activities such as cellulases sample from wild microbial strains and residual cellulase in the fermentation broth. Base on correlation the FPA values, e.g., a known commercial cellulase, and the HPLC analysis data, we could give equivalent FPA values of the low activity cellulase sample.

Measurement of the Ratio of Glucose to Cellobiose (G/C ratio) of the Enzyme Hydrolysis

The PFU assay using HPLC measurement provided the advantage of delivering glucose and cellobiose concentrations, instead of an overall reducing sugar concentrations by the DNS method. Based on the HPLC measurement data, the ratio of the released glucose (G) to the residual cellobiose (C), the G/C ratio, could be used as an important index for evaluating the cellulase quality. The high residual cellobiose, indicated by the low G/C ratio, is from the lack of β-glucosidase in the cellulase enzymes, and the cellobiose can rarely be utilized by the microorganisms. Figure 2 shows that even at the similar FPU loading of the three commercial enzymes, the G/C ratio of the different cellulase enzymes was significantly different. Accellerase 1000 would be more suitable for utilization in a practical process than the other two cellulases due to its capability of hydrolyzing cellulose to the fermentable glucose, instead of producing more cellobiose sugar. In contrast, the products in the filter paper hydrolysate produced by Spezyme CP and Youtell #5 contained greater cellobiose as shown in Fig. 2a. This was probably due to a shortage of cellobiase activity that then led to the incomplete conversion of cellobiose during the hydrolysis time.

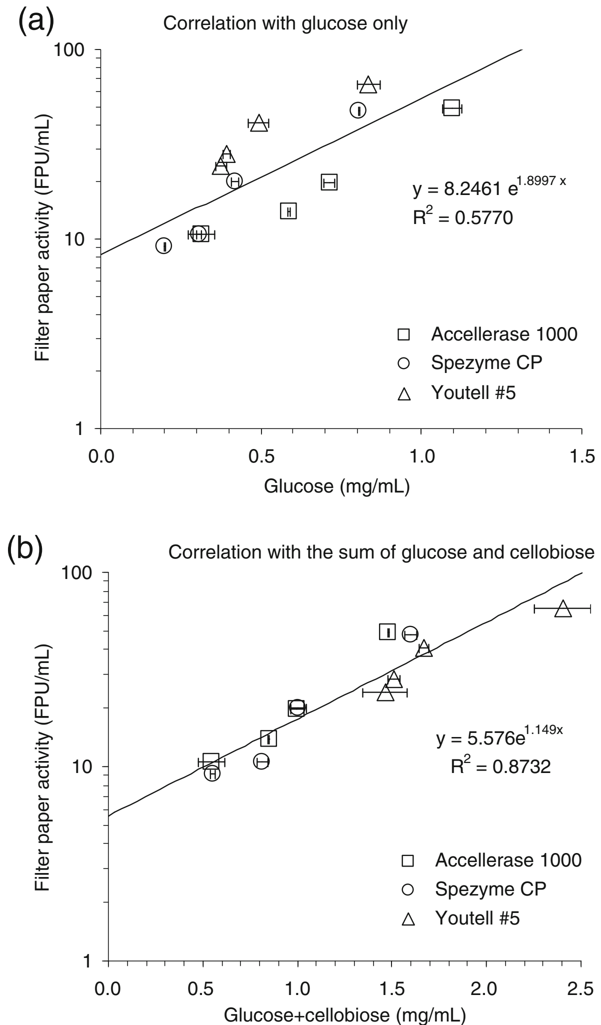
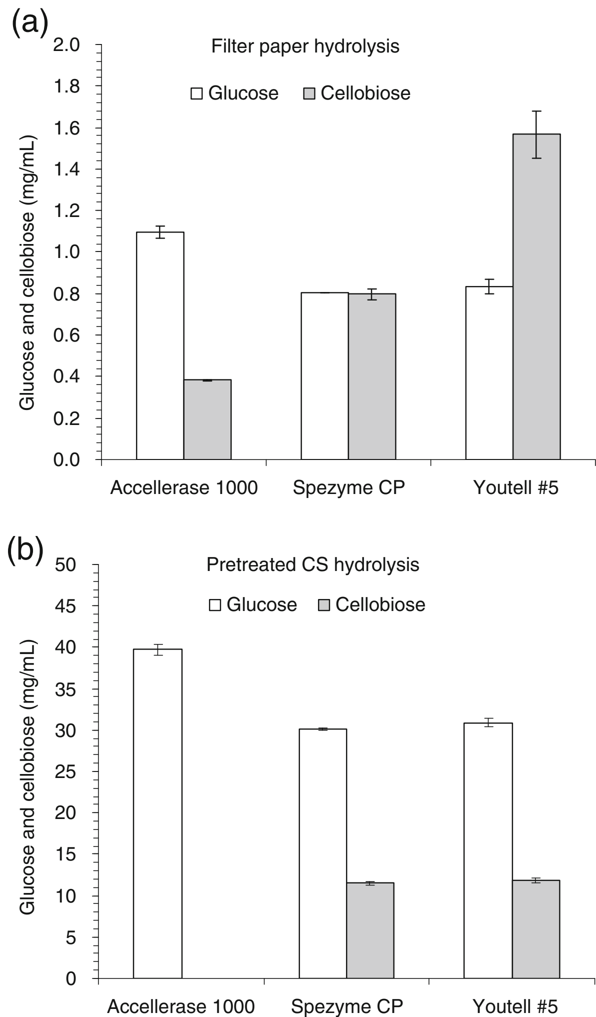


Fig. 1 Filter paper units determined by the standard FPA method versus the sugar concentration (s) using HPLC measurement. **a** Correlation with the glucose concentration only. **b** Correlation with the sum of glucose and cellobiose concentrations. Three commercial cellulases were diluted with citrate buffer to four dilutions (undiluted, onefold, twofold, and fourfold). The filter paper activity of each enzyme dilution was measured using a standard FPU assay method using DNS measurement. Each enzyme dilution was further diluted to 100-fold and then used for enzymatic hydrolysis of filter paper as described in "Materials and Methods"

Figure 2b shows the hydrolysis performance of the dilute acid pretreated CS for 24 h using three commercial cellulase enzymes at the equivalent FPU. The result indicates that the concentration profiles of glucose and cellobiose were significantly different. Only 40 g/L of glucose existed, and no cellobiose was left after 24 h of hydrolysis using Accellerase 1000. On the other hand, cellobiose accumulated was over 10 g/L, and glucose was less than 30 g/L when Spezyme CP and Youtell #5 were used. Thus, the use of the FPU value alone to characterize a cellulase was inadequate to illustrate the potential cellulolytic capacity of a cellulase. β-Glucosidase and other accessory enzymes can have a significant effect on cellulase performance [4, 12]. Using the HPLC-based FPU assay, the G/C ratio provided a useful and important index for evaluating the broader properties of cellulase enzymes. Johnston et al. [13] reported a method of cellulase activity assay using the bicinchoninic acid reagent, as in the case of DNS, for determining the reducing sugars. This method still involved problems like the control of color development and unspecific determination of

Fig. 2 Enzyme assay for hydrolysis of filter paper and the dilute acid pretreated CS using different cellulases. **a** Filter paper hydrolysis at the conditions described in "Materials and Methods." b Pretreated CS hydrolysis at 10 % (w/w) solid loading, 10 FPU/g dry mass, pH 4.8 citrate buffer in shake flasks at 50 °C with shaking at 150 rpm



Enzymes	Filter paper units (FPU/mL)	G/C ratio
Accellerase 1000	48.8 ± 0.5	2.87 ± 0.07
Spezyme CP	45.2 ± 2.7	1.01 ± 0.03
Youtell #5	63.7 ± 1.5	0.54 ± 0.02

sugars concentration. Hu et al. [14] reported using the quartz crystal microbalance technique for measuring the cellulase activity. This method used indirect estimation of the reducing sugars based on the solution viscosity and density changes in the enzymatic hydrolysis solution. Furthermore, the equipment used in the method might not be a regular machine and not be a routine method for measuring cellulase activity. Here the cellulase activity based on the HPLC method has the advantages of specific determination of sugars and practical applicability.

Conclusion

A simplified FPA method of cellulase enzymes was proposed based on the HPLC measurement in this study. The sum of glucose and cellobiose concentrations measured by HPLC was able to be linearly correlated with the FPU values. The FPU values of cellulase enzymes could be estimated by quantifying the glucose and cellobiose by HPLC. This method for FPU assay also provided another parameter of the ratio of glucose to cellobiose (G/C ratio) representing the potential capacity of cellulases converting cellulose to available monomeric sugars in the practical process.

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