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Quantification of bound and free enzymes during enzymatic hydrolysis and their reactivities on cellulose and lignocellulose

Zhiying Yu, Hasan Jameel, Hou-min Chang, Richard Philips, Sunkyu Park *

Department of Forest Biomaterials, North Carolina State University, Raleigh, NC 27695, USA

HIGHLIGHTS

- Bound enzymes are primarily responsible for enzymatic hydrolysis.
- Free enzymes become insignificant for enzyme hydrolysis after a certain time.
- The loss of synergistic effect in free enzymes limits digestibility of free enzymes.
- Tween 80 assists releasing CBH I into free enzymes.
- With surfactant, the digestibility of free enzymes on Avicel was greatly enhanced.

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ABSTRACT

Enzymatic hydrolysis of insoluble biomass is a surface reaction. Part of the enzyme adsorb on the surface of biomass, whereas the others stay in the liquid phase. In this study, three substrates (Avicel cellulose, bleached hardwood pulp, and green-liquor pretreated hardwood pulp) were used to study the reactivity of bound and free enzyme. In a continuous enzymatic hydrolysis, 35–65% initially added enzymes became bound enzymes, which were primarily responsible for enzymatic hydrolysis. The contribution from free enzymes became insignificant after a certain period of reaction time. SDS–PAGE analysis showed that CBH I was significantly decreased in the free enzyme, which might be the reason for the low digestibility of free enzymes due to the loss of synergistic effect. When Tween 80 was added during enzymatic hydrolysis, the digestibility of free enzyme on Avicel was greatly enhanced. However, the benefit of surfactant was not noticeable for lignocellulosic pulps, comparing to Avicel.

1. Introduction

The development of bioethanol and platform chemicals from lignocellulose is a promising potential alternative to our current reliance on fossil fuels (Chandra et al., 2007). A key issue for this process is the conversion of polysaccharides from lignocellulose into fermentable sugars. Therefore, enzymatic hydrolysis is a critical process not only because the sugar yields in the hydrolysates directly determine the efficiency of fermentation, but also the cost of enzymes has a great impact on the feasibility of bioethanol production in a commercial scale.

The efficiency of enzymatic hydrolysis of lignocellulose is governed by the chemical and physical properties of substrates, and the reactivity and synergistic effect of cellulase cocktails. Enzymatic hydrolysis of insoluble biomass is a heterogeneous reaction. Cellulases firstly adsorb onto solid substrate to cleave cellulose chains and release soluble cellobiose, which can be further hydrolyzed to glucose by β-glucosidase. In cellulase cocktails which were designed to have a synergistic effect, only a portion of the loaded enzymes adsorbs on the substrates as bound enzymes such as cellobiohydrolases (CBHs). The other portions of enzymes in the liquid phase, such as β-glucosidases and desorbed CBHs, are free enzymes which are potentially available for recycling. Enzymatic hydrolysis is a dynamic equilibrium process in which the concentrations of bound enzymes and free enzymes are always changing due to the amount of available substrate and its surface characteristics.

In the past two decades, a great progress has been achieved in understanding the mechanism of enzymatic hydrolysis, especially the adsorption of cellulase. The adsorption of enzymes onto carbohydrates involve hydrogen bonds, van der Waals interactions, and ionic polarization or hydrophobic interactions (Ding and Xu, 2004; Lehtio, 2003). The most common description of cellulase

* Corresponding author. Tel.: +1 919 515 0473; fax: +1 919 515 6302.
E-mail address: sunkyu_park@ncsu.edu (S. Park).

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adsorption is the Langmuir isotherm, which measures overall cellulase adsorption, assuming the cellulose surface is uniform, the adsorption enthalpy is independent on surface coverage, and there is negligible interaction among adsorbed molecules. However, the insoluble biomass is far from an ideal substrate model. It has a non-uniform surface due to its morphology, cellulose crystallinity, and heterogeneous components (*i.e.* hemicellulose and lignin). Therefore, the dynamic adsorption of cellulases with respect to the changes of substrate could be much more complicated. Researchers have studied the behavior of adsorbed cellulases in a dynamic process. It was found that adsorbed enzymes were sufficient to digest cellulose without replenishing the enzymes while the liquid phase containing the sugar and free enzymes was continuously removed (Mandels et al., 1971). Another research compared two scenarios of enzymatic hydrolysis without removing the supernatant and with replacement of the supernatant by fresh buffer and the same amount of produced glucose. They found that the free enzymes had little functions for hydrolysis (Lee and Fan, 1983). However, whether this observation was consistent with the heterogeneous enzyme cocktails and lignocellulosic materials was not yet reported. The importance of utilizing the bound enzymes should be further investigated.

From a process standpoint, enzymatic hydrolysates are used for fermentation while there are still substantial amount of enzymes in hydrolysates as free enzymes. β-Glucosidases together with some desorbed enzymes are the major components in free enzymes. Therefore, it is technically feasible to recover and reuse these free enzymes from hydrolysates. Some of the previous works showed that cellulases can be recovered in high yields by contacting fresh steam-exploded wood with hydrolysis filtrate, and recycled enzymes gave a hydrolysis rate about equal to those with fresh enzymes (Clesceri et al., 1985). Lu et al. found that the free enzymes recycled via ultrafiltration remained relatively active for three rounds of recycle (Lu et al., 2002). However, Hogan and Mes-Hartree found that the recovered enzymes had only 10–20% filter paper activity compared with the original enzymes. They suggested that the low enzyme activity of the recovered enzymes was due to the combination of thermal inactivation and adsorption of some of the cellulases onto the lignocellulosic residue (Hogan and Mes-Hartree, 1990). A recent study revealed that a difference in the lignin content between acid and alkali treated wheat straws could significantly affect cellulase adsorption and cellulase recycling efficiencies (Qi et al., 2011). The structural changes of lignin during pretreatment could even increase the non-productive adsorption of enzymes (Rahikainen et al., 2013).

Despite some significant progresses made so far, the digestibility of bound and free enzymes with the different types of substrates remain unclear. As the hydrolysis time progressed, whether the bound enzymes and free enzymes still possess most hydrolysis abilities and how the enzymes' digestibility changes with the reaction time needs to be understood. Based on these considerations, the objective of this research is to study the characteristics of bound and free enzymes during enzymatic hydrolysis and seek for the potentials of re-using bound and free enzymes for a more efficient and economical application. In this study, Avicel, bleached hardwood pulp, and alkaline-pretreated hardwood pulp, were used to evaluate the characteristics of bound and free enzymes. Understanding the nature and behavior of bound and free enzymes is of significant importance for enhancing the fundamental insights of enzymatic hydrolysis as well as bringing value to industrial applications, such as strategies for enzyme recycles and reuse (Xue et al., 2012a), and multistage enzymatic hydrolysis by removal the liquid stream of hydrolysate in each stage and splitting the addition of enzymes into each stage, which maximally uses the bound enzymes while decreasing the required operation volume of enzymatic hydrolysis (Xue et al., 2012b).

2. Methods

2.1. Materials

Microcrystalline cellulose (Avicel PH-101) was purchased from Sigma–Aldrich (St. Louis, MO). Fully bleached hardwood pulp (BHW) was obtained from a mill in the southeastern United States. The preparation of alkaline-pretreated hardwood pulp with green-liquor (GLHW) using the mixed southern hardwood (mixture of oak, maple, poplar, ash, and sweet gum) as the resources was conducted in the lab. Chemical compositions of these substrates were shown in Table 1. The green-liquor pretreatment was conducted with 16% total alkali charged green liquor, 40% sulfidity, 400 H-factor, and 160 °C, with a liquid-to-solid ratio of 4 (Yu et al., 2011). After green liquor pretreatment, the chips were fiberized using an 8-in. disk refiner and subjected to oxygen delignification with 3% sodium hydroxide at 110 °C for 1 h at an oxygen pressure of 100 psi. Then, the PFI refining was performed with 4000 revolution counts at 10% consistency. Three enzymes, including NS50013 (cellulase), NS50014 (hemicellulase), and NS50010 (β-glucosidase), were kindly provided by Novozymes North America, Inc. (Novozymes, Franklinton, NC).

2.2. Preparation of bound and free enzymes by pre-enzymatic hydrolysis

Avicel, BHW, and GLHW were hydrolyzed for 20 min, 3 h, 12 h, and 48 h at 5% consistency using 10 oven dried (OD) grams of substrate mixed with 100 mM sodium acetate buffer (pH 4.8) supplemented with 0.3% sodium azide. The pre-enzymatic hydrolysis (pre-EH) was conducted with an enzyme loading of 5 FPU/g of cellulase and a 0.3 weight ratio of β-glucosidase per gram of carbohydrate (6.3 mg proteins of cellulase and 3.6 mg proteins of β-glucosidase per gram of carbohydrate) at 50 °C with a shaking rate of 180 rpm. Another two scenarios, involving the addition of 15 mg/g substrate Tween 80 at the beginning of 12 h pre-EH and at 20 min before the end of 12 h pre-EH, respectively, was performed as well. The process flow diagrams are shown in Fig. 1. Duplicates of each sample were conducted for weight loss measurement and protein analysis. After pre-EH, the solid and liquid were separated using a Bush funnel with Whatman No. 1 filter paper. The soluble sugar in liquid was measured by HPLC and the proteins in a liquid were determined by ninhydrin assay. The solid residues were carefully removed from the filter paper. A small amount of the residues were used for moisture analysis and freeze-dried to measure the amount of un-hydrolyzed substrate and bound enzymes.

The digestibility of bound and free enzymes was investigated by kinetic hydrolysis. Replacing the same volume of filtrate with fresh buffer and continuing the hydrolysis for up to 144 h evaluated the digestibility of bound enzymes. For determination of the digestibility of free enzymes, parts of the filtrate were mixed with 5 OD g of fresh substrate at 5% consistency and continued the hydrolysis for up to 144 h. In the case of pre-EH supplemented by Tween 80, 12 h pre-EH was conducted on all the substrates with 15 mg/g substrate of Tween 80 added at the beginning of pre-EH, and 20 min prior to the end of the reaction, respectively. All the experiments were duplicated and the results were reported as average.

2.3. Direct quantification of the bound and free enzymes

The protein concentration of enzymes was measured by ninhydrin assay analysis. Bovine serum albumin (BSA) was used as reference standard. Protein solutions of 0.1 mL with a maximum protein concentration of 1 mg/mL were mixed with 0.3 mL