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Quantification of cellulase adsorbed on saccharification residue without the use of colorimetric protein assays

Hiroshi Nonaka[a,*], Akihiro Hideno[b]

[a] Graduate School of Bioresources, Mie University, 1577 Kurimamachiya-cho, Tsu, Mie 514-8507, Japan
[b] Graduate School in Faculty of Agriculture, Paper Industry Innovation Center, Ehime University, 127 Mendori-cho, Shikokuchuo, Ehime 799-0113, Japan

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ABSTRACT

Adsorption of cellulase onto the saccharification residues of lignocellulose, especially onto lignin, is unproductive and poses a significant problem for recycling of the enzyme. We developed a direct method to quantify residue-adsorbed cellulase by elemental analysis without use of calorimetric methods such as the Bradford, BCA, or Lowry assays. First, initial cellulase loading was estimated from the absorbance at 280 nm using the weight adsorption coefficient of cellobiohydrolase I (CBHI), predicted using the amino acid sequence. Secondly, small amounts of nitrogen derived from adsorbed cellulase were detected using an elemental analyzer by analyzing various large quantities of residue. The nitrogen content of the *Trichoderma reesei* cellulolytic enzymes was calculated to be 16.5–17.3% based on the amino acid sequences, and the quantity of residue-adsorbed cellulase was calculated from the nitrogen content of the residue, using the nitrogen content of CBHI (16.5%). For example, following enzymatic saccharification of ball-milled softwood for 72 h with Accellerase 1500 (25 filter paper units/g substrate), it was possible to estimate that 18–28 mg cellulase/g of residue was adsorbed onto the residues, indicating that 19–28% of the initially added cellulase would be difficult to recover because of adsorption.

1. Introduction

Cellulase is a key enzyme in biorefinery processes such as production of ethanol, lactic acid, and butanol from lignocellulosic biomass. Currently, the cost of the cellulase required for enzymatic hydrolysis significantly affects the price of cellulosic biomass-derived products. Therefore, the recycling of cellulase is considered important for decreasing the enzyme cost.

After enzymatic hydrolysis of lignocellulosic biomass, cellulase remains in the saccharification liquor, which contains the glucose product, or is bound to the residue [1–5]. Free cellulase can be recovered from the saccharification liquor by addition of fresh cellulosic substrate [4,6]. However, bound cellulase is partially irreversibly adsorbed to lignin, and is thus difficult to recover [7].

Therefore, it is important to accurately quantify the amount of residue-bound cellulase. Recent studies on cellulase adsorption have proposed a variety of pretreated cellulosic substrates [8,9] as well as isolated lignins, e.g., Bjorkman lignin [10], alkali lignin [11], cellulolytic enzyme lignin [6,12], and lignophenol [13]. However, the adsorbed cellulase protein has been indirectly quantified as the difference between the total quantity of protein initially added and the quantity of protein remaining in the liquor, primarily using colorimetric protein assays such as the Bradford [14], Lowry [15], or bicinchoninic acid (BCA) assays [16].

A major disadvantage of indirect colorimetric methods for measuring the quantity of adsorbed cellulase protein is the generation of false-positive results due to surfactants and soluble compounds extracted or produced from the lignocellulosic substrate. For example, the results of the BCA method were shown to be affected by the presence of xylose, glucose, and cellobiose, which resulted in false positives. In particular, the effect of xylose was stronger than that of glucose or cellobiose [17]. Removal of interfering compounds from the hydrolysis liquor by ultrafiltration methods, such as VIVASPIN treatment, prior to applying colorimetric protein assays may help to prevent false positives. However, such ultrafiltration processes are complicated and may result in material loss and other errors. Another fundamental drawback of colorimetric protein assays is differences in coloring between the target protein and the standard protein used [18], which may result in serious misreading of the absolute protein concentration. Therefore, the development of a direct method for the quantification of residue-adsorbed cellulase that does not depend on colorimetric methods is required, particularly for use during or after enzymatic hydrolysis of lignocellulosic biomass.

* Corresponding author. Tel.: +81 59 231 9520; fax: +81 59 231 9591.
E-mail address: nonaka@bio.mie-u.ac.jp (H. Nonaka).

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Recently, the Wyman research group has applied nitrogen content measurement to detect cellulase on solid substrates. Cellulase adsorption on Avicel [19], corn stovers pretreated with dilute acid with or without Tween 80 [20], and poplar pretreated with AFEX, ARP, dilute acid, and lime [21] was monitored by measuring the nitrogen content of the substrate. The nitrogen content was converted to the cellulase adsorption capacity using a typical nitrogen-to-protein conversion factor (nitrogen factor), 6.25 [19,20]. The method was improved by reexamining the nitrogen factors for several commercial cellulases, which range 7.90–8.40, indicating that the nitrogen content ranged 11.9–12.4% [17]. These newly established nitrogen factors have since been utilized to convert the nitrogen content of solids into the quantity of adsorbed protein [8,22]. However, nitrogen content of approximately 12% appears to be underestimates of the nitrogen content of real protein without stabilizers. These low estimates may have resulted from the use of the BCA assay for protein quantification. In the same report [17], the nitrogen content, calculated from the amino acid composition of cellobiohydrolase I (CBHI), cellobiohydrolase II (CBHII), and endoglucanase I (EGI) of *Trichoderma reesei*, was shown to be 13.7%, 13.9%, and 13.7%, respectively. These values are corresponding to the nitrogen content calculated from total amino acids; however, the actual values should be much higher because the molecular mass is decreased by dehydration accompanied by formation of peptide bonds.

Most recently, other researchers have begun to use nitrogen content to study the inhibitory effect of lignin on cellulose bioconversion [23]. Monitoring nitrogen itself appears to provide a functional of cellulase protein content; however, methods for quantification of the residue-adsorbed cellulase require further investigation. If a successful quantification method could be developed, data could be accumulated on the behavior of lignocellulosic residue-binding cellulase formed during the enzymatic hydrolysis of various cellulosic substrates.

In this study, we performed enzymatic saccharification of ball-milled Japanese cypress (BMJC) (*Chamaecyparis obtusa*) to address this. Japanese cypress (JC) is a representative softwood grown in Japan. Following enzymatic saccharification, we separated the saccharification residue and liquor by centrifugation, and quantified the residue-adsorbed cellulase using a newly developed direct method based on elemental analysis.

2. Materials and methods

2.1. Substrate

Wood chip samples of JC were electrically sieved to collect chips ranging in size from 125 μm to 500 μm. Monomeric sugar and lignin contents of JC were analyzed using the Technical Association of the Pulp and Paper Industry (TAPPI) Test Method T249 [24] and determined as follows (in terms of dry weight per gram of JC): 449 mg glucose, 44 mg xylose, 105 mg mannose, 31.9% acid-insoluble lignin (Klason lignin), and 1.1% acid-soluble lignin. Approximately 1 g of sieved sample was ground at 400 rpm for 20 min using a planetary ball mill (P-6, FRITSCH Co., Grunwald, Germany) operating with approximately 118 g of stainless steel balls (diameter, 10 mm).

2.2. Enzymatic hydrolysis of BMJC

Accellerase 1500 (ACC1500; Genencor, Rochester, NY, USA) was centrifuged and the supernatant was used for enzymatic hydrolysis following the method of Inoue et al. [25]. The ball-milled samples (dry weight: 0.0515 ± 0.0010 g) and 1.0 mL of

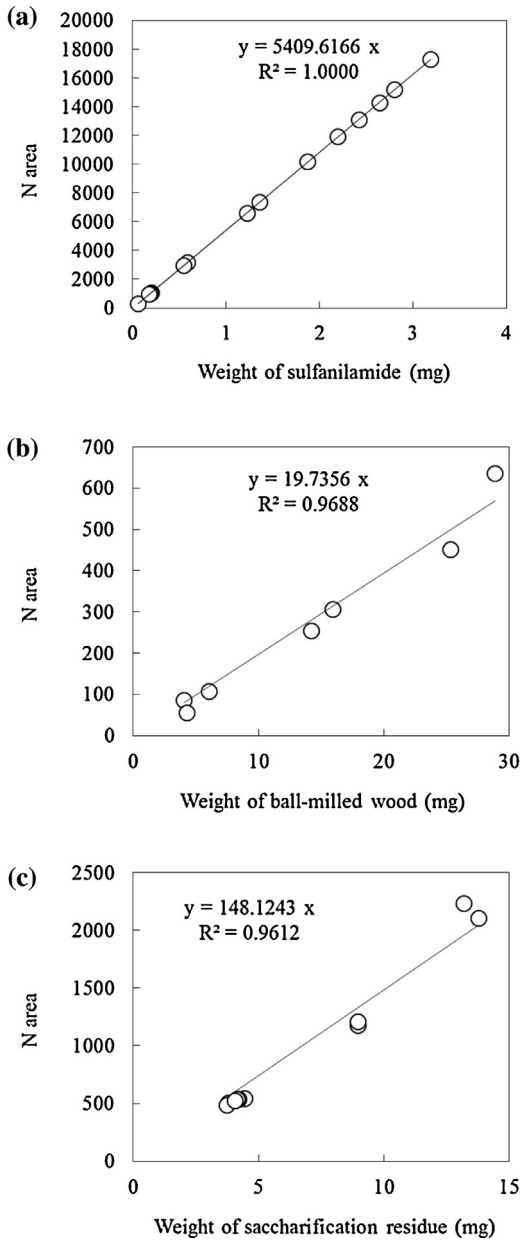

Fig. 1. Relationship between sample weight and nitrogen peak area using an elemental analyzer. (a) Sulfanilamide, (b) ball-milled Japanese cypress and (c) residue after enzymatic hydrolysis for 72 h.

17 μL/mL ACC1500 (approximately 1.3 filter paper unit/mL, prepared with 50 mM acetate buffer (pH 5.0)) were placed in 2-mL tubes and incubated at 45 °C for 2 h with agitation using a rotator.

2.3. Measurement of nitrogen content of residues by elemental analysis

After hydrolysis, the samples were centrifuged (20,000 × *g* for 10 min) and the resulting residues were washed with 1 mL of water and re-centrifuged (20,000 × *g* for 10 min); the supernatants were discarded. This washing step was repeated three times. The washed residues were freeze-dried for elemental analysis. Elemental analysis was performed to detect nitrogen derived from small amounts of cellulase protein adsorbed onto the residue (vario EL cube; Elementar, Hanau, Germany).