The enzymatic hydrolysis rate of cellulose decreases with irreversible adsorption of cellobiohydrolase I

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ABSTRACT

Protein adsorption onto solid substrates usually takes place in an irreversible fashion and this irreversible adsorption also occurs in some enzymatic reactions. In this work the adsorption behavior of intact β -1, 4-glucan-cellobiohydrolase (CBH I) from *Trichoderma reesei* onto microcrystalline cellulose was monitored by surface plasmon resonance and UV-spectral method. It was found that there existed reversible binding and irreversible binding of CBH I during its interaction with cellulose substrate. To evaluate the influence of adsorption on cellulose enzymatic hydrolysis, the reaction dynamics on pure cellulose were determined. A plot of the hydrolysis rate against the surface density of irreversibly adsorbed CBH I, revealed an inverse relationship in which an apparent decrease in the hydrolysis rate was observed with increasing surface density. Taken together, results presented here should be useful for modifying the binding characteristics of CBH I and making them more effective in cellulose hydrolysis.

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1. Introduction

The adsorption of proteins at a solid–liquid interface is a common phenomenon which has been widely observed in various areas such as biology, medicine, biotechnology, and food processing [1,2]. Proteins are often considered to be denatured at solid–liquid interface [1], and it has been assumed that the extent to which a protein becomes denatured is correlated with its irreversible adsorption to the interface [3]. The irreversible protein adsorption is usually unfavorable in biocatalysis, where the irreversibly bound proteins occupy a large number of surfaces while limiting the access of other functional proteins. On the other hand, the irreversibility of protein adsorption should have a great effect on the downstream processing. Regarding its important implications in medicine, biotechnology, and food processing, the irreversibility of protein adsorption on a solid surface has been extensively studied [3–5]. However, much less attention has been paid to studying the enzyme adsorption at its substrate solid–liquid interface such as in the case of cellulases acting on insoluble cellulose.

Adsorption of cellulases to the substrate cellulose surface is the first step in the enzymatic hydrolysis of cellulose. Unlike enzymatic hydrolysis with soluble substrates, adsorption of cellulases takes place at cellulose–liquid interface. Various studies on fungal cellulases have shown that once adsorbed, the desorption of the enzyme is not easy and that the binding is, at least, partly irreversible [6–9]. Adsorption and its relation to cellulose hydrolysis has been the focus of many studies. Especially, the phenomenon that the rate of cellulose hydrolysis declines dramatically as hydrolysis proceeds, called rate retardation, has been observed by many investigators [10,11]. It has been suggested that the rate of hydrolysis is proportional to the amount of adsorbed enzymes [12,13]. However, recent studies using a single purified cellulase enzyme β -1, 4-glucan-cellobiohydrolase (CBH I) (EC 3.2.1.91) and a homogeneous substrate showed that a declining rate of reaction was still observed at the initial stage of adsorption [14].

The reversibility and irreversibility of adsorption of CBH I onto insoluble cellulose has been demonstrated in previous studies. However, information on the role of irreversible adsorption of CBH I during cellulose hydrolysis is far from clear. On the other hand, most research has focused on the relationship between the rate retardation and the total adsorption, much less attention has been paid to how reversibility and irreversibility of adsorption relating with rate retardation. We, therefore, tried to understand the influence of irreversible adsorption CBH I on hydrolysis rate during the hydrolytic process from this point of view. In this study, the adsorption behavior of CBH I onto cellulose CF11 was monitored by

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UV-spectral method and surface plasmon resonance (SPR) which is an optical method that is usually used to register the protein mass adsorbed.

2. Materials and methods

2.1. Purification of cellulase

CBH I was purified from a commercial cellulase, Celluclast (Novo Nordisk, Tianjin, China). Before purification, Celluclast samples were ultrafiltered with a Millipore microconcentrator fitted with a 10 kDa cut-off membrane (catalog number: PXB010A50, Millipore) and suspended in an exchange buffer. The exchange buffer was 50 mM sodium acetate buffer (pH 5.6). Ion exchange chromatography steps were performed as described previously [15,16]. The purity of fractions containing CBH I enzyme was confirmed by SDS-PAGE. Fractions with CBH I were also analyzed by measuring the activity against *p*-nitrophenyl β -D-cellobiose (*p*-NPC). The CBH I protein concentration was determined by absorbance measurements at 280 nm using a molar absorption coefficient of $78,800 \text{ M}^{-1} \text{ cm}^{-1}$ [17].

2.2. Adsorption of CBH I

Adsorption of CBH I on cellulose was monitored by the UV-spectral method and SPR, respectively. CF11 (Whatman), used as the substrate, was prepared as follows: the cellulose was washed thoroughly with distilled water by repeated steps of centrifugation and resuspension, and then freeze-dried. Low protein binding tubes (catalog number: 508-GRDS-Q, Sangon) with CF11 suspended in 50 mM sodium acetate buffer (pH 5.0) were preincubated at the hydrolysis temperature of 40°C for 60 min. Hydrolysis was carried out at 40°C in a shaking bath at 200 rpm. Enzyme concentration was $2.0\text{--}3.0 \mu\text{M}$. The reaction was started by adding CBH I at 40°C to 1.0 ml of CF11 suspension (1.0 mg/ml). At indicated time points, the hydrolysate was centrifuged ($10,000 \text{ rpm} \times 1 \text{ min}$), washed repeatedly with the same buffer as the buffer in the sample pre-incubated at 40°C , and all the supernatants were collected. The protein concentration in the supernatant was determined as described above. The mass of totally adsorbed CBH I was calculated by subtracting the mass of free CBH I in the first supernatant from the mass of the added CBH I. The CBH I still remaining on the substrate after repeated washing steps as the above mentioned protocol was regarded as irreversibly adsorbed enzyme. The mass of irreversibly adsorbed CBH I was calculated by subtracting the total mass of free CBH I in all the following supernatants from the mass of the added CBH I. The surface area of the cellulose was estimated by microscopic observation as previously described [18].

SPR was performed to confirm the UV-spectral method. CBH I adsorption onto the surface of CF11 was monitored in real time by the Biacore X system (Biacore AB, Uppsala, Sweden) at 40°C . 50 mM sodium acetate buffer, pH 5.0, was the buffer used in all SPR experiments and the buffer was prepared by filtering through a syringe filter (Millex GP, PES membrane, pore size $0.22 \mu\text{m}$, diameter 25 mm, Millipore) and ultrasonically degassing under vacuum. Immobilization of cellulose on the SPR chip surface was performed according to the affinity between biotin and streptavidin. Biotinylated cellulose was prepared using the procedure with slight modification [19]. Streptavidin was immobilized onto the CM5 chip surface using an EDC/NHS-mediated amine coupling protocol [20]. CBH I was introduced to the chip surface by passing through a series volume of CBH I ($100 \mu\text{M}$) samples in buffer during the experiments. The chip surface was automatically rinsed with the running buffer after all the samples were completely passed through the chip. Adsorption data were automatically collected by the Biacore X instrument and the experiment was performed thrice. The total adsorbed mass of CBH I was measured at a point immediately before washing with the buffer. After elution with the running buffer, the amount of CBH I that remained on the cellulose surface represented the irreversibly adsorbed mass [3].

2.3. Analysis of cellobiose production and reducing ends

The hydrolysate solution containing cellobiose was boiled for 10 min in a water bath followed by filtration through syringe filters (Millex GP, Millipore). Sample blanks (only substrate in solution without enzyme) were subjected to the same conditions as the experimental samples. Cellobiose, the hydrolysis product, was analyzed by HPLC [21]. The HPLC system (Shimadzu, Japan) was equipped with an Aminex HPX-87H column (Bio-Rad Laboratories). The mobile phase was 0.5 mM sulfuric acid (filtered through a $0.22 \mu\text{m}$ pore size membrane) eluting at a 0.4 ml/min flow rate. During operation, the column temperature was kept at 55°C . Standard cellobiose was used to make a calibration curve in order to calculate the concentration of cellobiose that was produced. In the meantime, changes of cellulose reducing ends (substrate concentration) were estimated at various time points of incubation by the BCA method as described by Zhang and Lynd [22].

2.4. CBH I activity assays and circular dichroism

p-NPC was used as the substrate for assays to measure the enzymatic activity of CBH I [23]. Enzymatic activities of CBH I desorbed upon washing with buffer and

CBH I irreversibly adsorbed on cellulose were measured. CBH I irreversibly adsorbed on cellulose was resuspended in buffer with substrate *p*-NPC when assays of activity. The experiment was performed at 40°C in a water bath, and the mixture was shaken every 5 min. The reaction was terminated by the addition of $1\% \text{ Na}_2\text{CO}_3$. Aliquots were transferred to a microtiter plate after centrifugal sedimentation ($10,000 \text{ rpm} \times 1 \text{ min}$) of the cellulose. The released *p*-nitrophenol was determined by measuring absorbance at 400 nm. Specific activities were recorded in terms of the release rate of *p*-nitrophenol in $\mu\text{mol/min}$. CBH I activities in different states were compared to assess the effects of irreversible adsorption on biological activity.

Circular dichroism (CD) spectra ($200\text{--}250 \text{ nm}$) of different treatments of CBH I were performed on a JASCO (J-810) circular dichroism spectrometer [24]. Spectra were recorded in 1 cm path length quartz cuvettes with a scan rate of 500 nm/min and a lower number of spectra. Samples of irreversibly adsorbed CBH I were selected from different indicated time points. The solution of CBH I was also measured and compared with that of resuspended solution of irreversibly adsorbed CBH I onto cellulose at identical conditions.

3. Results

3.1. Adsorption isotherms

Adsorption of CBH I on cellulose was monitored by means of both the UV-spectral method and SPR, respectively. The adsorbed behavior of CBH I and trends of adsorption isotherms were determined by the UV-spectral method and were found to be in close agreement with the results of SPR (Figs. 1 and 2). The enzyme adsorbed immediately to the substrate after being introduced to the reaction system. The initial part of the adsorption isotherm suggested that the enzyme had a high affinity for cellulose. After washing, part of the originally added CBH I still remained bound to cellulose, displaying an obviously irreversible adsorption onto the cellulose surface (Fig. 2). The trend of irreversible adsorption was similar to that of total adsorption as determined by different methods. However, the amount of adsorbed protein did not show a clear plateau, instead the percentage of irreversibly adsorbed enzyme to totally adsorbed amount showed a slow increase.

3.2. Hydrolysis of cellulose

The time course for changes in cellobiose concentration during hydrolysis of cellulose was investigated for different concentrations of CBH I. The hydrolytic rate is defined as dC/dt , where C is the concentration of the main hydrolysis product of cellobiose. The

Fig. 1. Adsorption isotherms of CBH I onto cellulose surface measured by conventional spectra method. Cellulose (1.0 mg/ml) was incubated at 40°C in 50 mM sodium acetate, pH 5.0, with $3.0 \mu\text{M}$ CBH I. total adsorption (□); irreversible adsorption (○).

Fig. 2. Adsorption isotherms of CBH I onto cellulose surface measured by SPR. Arrows denote amount of irreversibly adsorbed CBH I at indicated time points.

hydrolytic rate started to decline upon the adsorption of CBH I, and decreased dramatically until less than 10% of the initial hydrolysis rate remained after being incubated for 10 min with 2 μM of CBH I (Fig. 3). The trend showing a declining hydrolytic rate during hydrolysis was similar for various initial concentrations (2 and 3 μM) of CBH I in the reaction system (Fig. 3).

The possible influence of substrate concentration (reducing ends of cellulose) on hydrolysis rate was examined by measuring changes of reducing ends of residual cellulose at various time points of incubation by the method mentioned in Section 2. Absorbance at 560 nm hardly changed during the hydrolysis process indicating that the amounts of reducing ends present of residual cellulose remained nearly constant during hydrolysis (Fig. 3, inset).

Fig. 3. Hydrolysis of cellulose with CBH I. Cellulose (1.0 mg/ml) was incubated at 40 °C in 50 mM sodium acetate, pH 5.0, with 3.0 μM CBH I (○) and 2.0 μM CBH I (□). Inset: Reducing ends of cellulose during hydrolysis process with 3.0 μM CBH I were measured at selected time points by BCA assay.

Fig. 4. Surface density of CBH I adsorption versus hydrolysis rate. Cellulose (1.0 mg/ml) was incubated at 40 °C in 50 mM sodium acetate, pH 5.0, either with 2.0 μM of CBH I (A) or with 3.0 μM of CBH I (B). Surface density of total adsorption was determined by (□), surface density of irreversible adsorption was measured by (○).

In order to investigate the correlation between the hydrolysis rate and adsorption, the reaction dynamics on cellulose were determined in the same reaction system. Adsorption was evaluated by UV-spectral method during the hydrolysis process as described in Section 2. The cellulose hydrolysis rate treated by natural logarithm was plotted versus surface density of CBH I adsorption (i.e., the amount per unit surface area) (Fig. 4). While the cellulose hydrolysis rate for various CBH I concentrations was high at low surface density of adsorption, it declined with increasing surface density of irreversible adsorption.

3.3. Properties of irreversibly adsorbed CBH I

p-NPC is the widely used choice of substrate for assaying the enzymatic activity of CBH I (Fig. 5). To test our enzymatic activities of irreversibly adsorbed CBH I, samples were selected according to the adsorption isotherm. Compared with the reversibly desorbed enzymes at various time points during the process of the reaction, which still had nearly the same specific activity as originally added CBH I, the activities of irreversibly adsorbed CBH I on cellulose

Fig. 5. Relative specific enzymatic activity of CBH I. Cellulose (1.0 mg/ml) was incubated at 40 °C in 50 mM sodium acetate, pH 5.0, with 3.0 μ M of CBH I. Relative activities were measured at different time points during hydrolysis using *p*-NPC (0.5 mM in 50 mM sodium acetate, pH 5.0) as a substrate. Samples were selected and measured according to the adsorption isotherm: (A) 10 min time point; (B) 20 min time point; (C) 30 min time point. The maximum enzymatic activity in solution originally added was taken as 100%.

decreased dramatically. The relative specific activity of irreversibly adsorbed CBH I decreased by 70% after 10 min, and the activity decreased gradually as hydrolysis proceeded. Comparison of different CD spectra graphs for irreversibly adsorbed CBH I indicated that apparently different conformations of CBH I were adopted during the hydrolysis of cellulose (Fig. 6). Interestingly, changes really exist in the CBH I conformation and its specific activity during irreversibly adsorbed on cellulose with hydrolysis proceeding.

4. Discussion

Although an understanding of the connection between binding and hydrolysis is crucial in cellulase study, decisive conclusions have been elusive so far [8]. In this study, we focused on different

Fig. 6. Far-UV CD spectra of CBH I. Native CBH I in solution (solid line), irreversible adsorption of CBH I onto cellulose at 10 min time point (dashed line), at 30 min time point (dotted line).

aspects of binding and hydrolysis from a dynamic point of view. We showed that there existed reversible binding and irreversible binding during the CBH I adsorption onto and hydrolysis of cellulose. Importantly, an inverse relationship was uncovered between the surface density of irreversibly adsorbed CBH I and the hydrolysis rate during cellulose hydrolysis.

When cellulases hydrolyze cellulose, the cellulase–cellulose complex should consist of the soluble enzyme solution and the insoluble substrate cellulose. Obviously, cellulase enzymes have to act at the solid–liquid interface during the hydrolysis of cellulose. In this context, physicochemical properties of cellulases should be in accordance with the general rule of adsorption of protein at solid–liquid interface. Indeed, we observed the irreversible adsorption of CBH I onto cellulose by different methods (Figs. 1 and 2). Similar phenomena of irreversible adsorption, albeit under different conditions, were mentioned by Kyriacou et al. and Nidetzky et al. [7,9]. The most common description of cellulase adsorption is the Langmuir isotherm, assuming that adsorption can be described by a single adsorption equilibrium constant and a specified adsorption capacity [18]. However, our research showed that adsorption of CBH I onto cellulose is partially irreversible which does not comply with assumptions implicit in the Langmuir model. The binding parameters of our adsorption isotherms could not be fitted very well by an adaptation of the Langmuir model, which means that the adsorption of CBH I onto cellulose is not a simple phenomenon as described elsewhere.

Typical consideration about cellulase adsorption and cellulose hydrolysis is that there must be an initial stage where both binding and activity increase in parallel, which has found support in many reports [12,13]. However, these data were mostly obtained by use of cellulase mixtures. By using single CBH I, both data from Valjamae et al. [14] and our research in this study show that a declining rate happens initially during the enzyme adsorption stage. One reason for this discrepancy may be due to the very limited “productive” binding compared with much more “non-productive” binding. Another reason may lie in the fact that hydrolysis rate should be proportional to the concentration of substrate as the first order reaction. In this respect, since CBH I hydrolyzes a cellulose chain from the reducing end and moves along the same cellulose chain [25,26], changes of the actual number of reducing ends in cellulose might also affect the hydrolysis rate. However, reducing ends cannot be changed by using a single CBH I (Fig. 3, inset). Nonetheless, the amount of accessible reducing ends would decrease with irreversible binding. On the contrary, in the case of cellulase complex, endoglucanase can increase new reducing ends by acting on amorphous region of cellulose until it is hindered by crystal region. This may explain why a declining rate was not observed for the cellulase mixtures while it existed at the initial stage of adsorption by using a single CBH I.

During the degradation of insoluble cellulose, only absorbed enzymes can be considered “working enzymes”. Among absorbed enzymes, the specific activity of the reversible enzymes at various time points in the reaction system was nearly the same as that of CBH I applied in the reaction initially. But enzyme activities of the irreversibly adsorbed CBH I on cellulose changed dramatically (Fig. 5). This indicated that it is irreversibly adsorbed CBH I, but not the total adsorption contributes to the decreasing hydrolysis rate although a similar correlation also exists if plotted for that of total adsorption. Importantly, CD spectra results indicated that the conformation of CBH I in irreversible binding changed (Fig. 6), and they may be transferred into an unproductive state as indirectly supported by the decline of their specific activity.

Compared with the effort to explain the rate of retardation from the point of productive and unproductive status of CBH I, our results suggested that the declined hydrolysis rate could be correlated with

the increase of irreversible adsorption considering the inseparable relationship between adsorption and hydrolysis process. Nevertheless, we did not rule out the possibility that there might exist a productive status in those irreversible bound enzymes, and the possibility that those active reversible binding enzymes contributes to the low catalytic activity in the range of several minutes to hours although hydrolysis rate become very low as compared with that at the initial stage. Further research on mechanisms of irreversible adsorption of cellulases at cellulose solid–liquid interface would be helpful for understanding cellulase rate retardation. We believe that this may provide helpful information for engineering cellulase enzymes and altering the characteristics of CBH I, making it more effective and increasing the hydrolysis rate.

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References

- [1] Gray JJ. The interaction of proteins with solid surfaces. *Curr Opin Struct Biol* 2004;14:110–5.
- [2] Nakanishi K, Sakiyama T, Imamura K. On the adsorption of proteins on solid surfaces, a common but very complicated phenomenon. *J Biosci Bioeng* 2001;91:233–44.
- [3] Karlsson M, Ekeröth J, Elwing H, Carlsson U. Reduction of irreversible protein adsorption on solid surfaces by protein engineering for increased stability. *J Biol Chem* 2005;280:25558–64.
- [4] Sakiyama T, Tanino K, Urakawa M, Imamura K, Takahashi T, Nagai T, et al. Characteristics of tryptic fragments of bovine β -lactoglobulin on a stainless steel surface. *J Biosci Bioeng* 1999;88:536–41.
- [5] Su TJ, Lu JR, Thomas RK, Cui ZF, Penfold J. The adsorption of lysozyme at the silica–water interface: a neutron reflection study. *J Colloid Interface Sci* 1998;203:419–29.
- [6] Otter DE, Munro PA, Scott GK, Geddes R. Desorption of *Trichoderma reesei* cellulase from cellulose by a range of desorbents. *Biotechnol Bioeng* 1989;34:291–8.
- [7] Kyriacou A, Neufeld RJ, MacKenzie CR. Reversibility and competition in the adsorption of *Trichoderma reesei* cellulase components. *Biotechnol Bioeng* 1989;33:631–7.
- [8] Palonen H, Tenkanen M, Linder M. Dynamic interaction of *Trichoderma reesei* cellobiohydrolases Cel6A and Cel7A and cellulose at equilibrium and during hydrolysis. *Appl Environ Microbiol* 1999;65:5229–33.
- [9] Nidetzky B, Steiner W, Claeysens M. Cellulose hydrolysis by the cellulases from *Trichoderma reesei*: adsorptions of two cellobiohydrolases, two endoglucanases and their core proteins on filter paper and their relation to hydrolysis. *Biochem J* 1994;303:817–23.
- [10] Desai SG, Converse AO. Substrate reactivity as a function of the extent of reaction in the enzymatic hydrolysis of lignocellulose. *Biotechnol Bioeng* 1997;56:650–5.
- [11] Eriksson T, Karlsson J, Tjerneld F. A model explaining declining rate in hydrolysis of lignocellulose substrates with cellobiohydrolase I (cel7A) and endoglucanase I (cel7B) of *Trichoderma reesei*. *Appl Biochem Biotechnol* 2002;101:41–60.
- [12] Sattler W, Esterbaue H, Glatter O, Steiner W. The effect of enzyme concentration on the rate of hydrolysis of cellulose. *Biotechnol Bioeng* 1989;33:1221–34.
- [13] Steiner W, Sattler W, Esterbaue H. Adsorption of *Trichoderma reesei* cellulase on cellulose: experimental data and their analysis by different equations. *Biotechnol Bioeng* 1988;32:853–65.
- [14] Valjamae P, Sild V, Pettersson G, Johansson G. The initial kinetics of hydrolysis by cellobiohydrolases I and II is consistent with a cellulose surface–erosion model. *Eur J Biochem* 1998;253:469–75.
- [15] Boer H, Teeri TT, Koivula A. Characterization of *Trichoderma reesei* cellobiohydrolase Cel7A secreted from *Pichia pastoris* using two different promoters. *Biotechnol Bioeng* 2000;69:486–94.
- [16] Medve J, Lee D, Tjerneld F. Ion-exchange chromatographic purification and quantitative analysis of *Trichoderma reesei* cellulases cellobiohydrolase I, II and endoglucanase II by fast protein chromatograph. *J Chromatogr A* 1998;808:153–6.
- [17] Stahlberg J, Johansson G, Pettersson G. *Trichoderma reesei* has no true exocellulase: all intact and truncated cellulases produce new reducing end groups on cellulose. *Biochim Biophys Acta* 1993;1157:107–13.
- [18] Zhang YH, Lynd LR. Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems. *Biotechnol Bioeng* 2004;88:797–824.
- [19] Li FC, Qi X, Geng MY. A novel method of immobilization of 911 for surface plasmon resonance biosensor. *Chin J Anal Chem* 2003;31:266–70.
- [20] Cooper MA. Label-free screening of bio-molecular interactions. *Anal Bioanal Chem* 2003;377:834–42.
- [21] Medve J, Karlsson J, Lee D, Tjerneld F. Hydrolysis of microcrystalline cellulose by cellobiohydrolase I and endoglucanase II from *Trichoderma reesei*: adsorption, sugar production pattern, and synergism of the enzymes. *Biotechnol Bioeng* 1998;59:621–34.
- [22] Zhang YH, Lynd LR. Determination of the number-average degree of polymerization of cellobiohydrolases and cellulose with application to enzymatic hydrolysis. *Biomacromolecules* 2005;6:1510–5.
- [23] Deshpande MV, Eriksson KE, Pettersson LG. An assay for selective determination of exo-1,4-beta-glucanases in a mixture of cellulolytic enzymes. *Anal Biochem* 1984;138:481–7.
- [24] Koutsopoulos S, Tjeerdema AM, Lieshout JF, van der Oost J, Norde W. In situ structure and activity studies of an enzyme adsorbed on spectroscopically undetectable particles. *Biomacromolecules* 2005;6:1176–84.
- [25] Teeri TT, Koivula A, Linder M, Wohlfahrt G, Divne C, Jones TA. *Trichoderma reesei* cellobiohydrolases: why so efficient on crystalline cellulose? *Biochem Soc Trans* 1998;26:173–8.
- [26] Teeri TT. Crystalline cellulose degradation: new insight into the function of cellobiohydrolases. *Trends Biotechnol* 1997;15:160–7.