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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 homogenous 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 M^{-1} 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 2.0–3.0 μM. 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 rpm × 1 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 μm, 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 μM) 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 μm 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% Na_2CO_3. Aliquots were transferred to a microtiter plate after centrifugal sedimentation (10,000 rpm × 1 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 μmol/min. CBH I activities in different states were compared to assess the effects of irreversible adsorption on biological activity.

Circular dichroism (CD) spectra (200–250 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

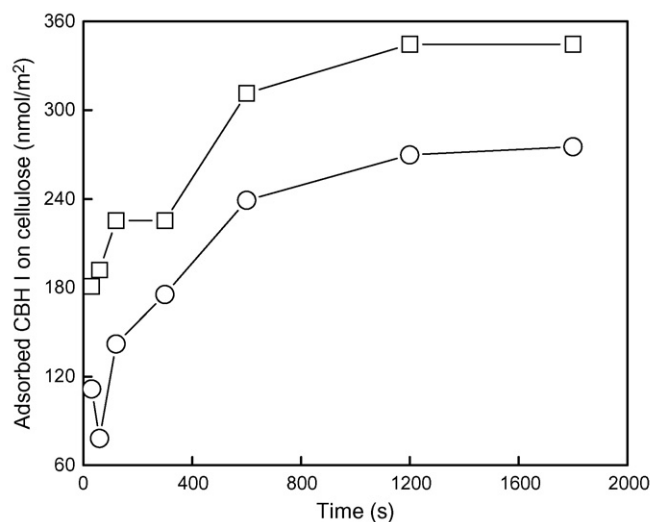

Fig. 1. Adsorption isotherms of CBH I onto cellulose surface measured by conventional spectra method. Cellulose (1.0 mg/ml) was incubated at 40 °C in 50 mM sodium acetate, pH 5.0, with 3.0 μM CBH I. total adsorption (□); irreversible adsorption (○).

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