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Description of Cellobiohydrolases Ce16A and Ce17A from *Trichoderma reesei* Using Langmuir-type Models

Dong Won Kim* and Young Gwan Hong

Department of Chemistry, Physical Chemistry Laboratory, College of Natural Sciences, Chungbuk National University, Cheongju 361-763, Korea

Abstract The binding of cellobiohydrolases to cellulose is a crucial initial step in cellulose hydrolysis. In the search for a detailed understanding of the function of cellobiohydrolases, much information concerning how the enzymes and their constituent catalytic and cellulose-binding changes during hydrolysis is still needed. The adsorption of purified two cellobiohydrolases (Ce17A and Ce16A) from *Trichoderma reesei* cellulase to microcrystalline cellulose has been studied. Cellobiohydrolase II (Ce16A) does not affect the adsorption of cellobiohydrolase I (Ce17A) significantly, and there are specific binding sites for both Ce17A and Ce16A. The adsorption affinity and tightness of the cellulase binding domain (CBD) for Ce17A are larger than those of the CBD for Ce16A. The CBD for Ce17A binds more rapidly and tightly to Avicel than the CBD for Ce16A. The decrease in adsorption observed when the two cellobihydrolases are studied together would appear to be the result of competition for binding sites on the cellulose. Ce17A competes more efficiently for binding sites than Ce16A. Competition for binding sites is the dominating factor when the two enzymes are acting together, furthermore adsorption to sites specific for Ce17A and Ce16A, also contributes to the total adsorption.

Keywords: activation energy, adsorption rate constant, binding capacities, cellulase, cellulose

INTRODUCTION

The cellulolytic enzyme system of *Trichoderma reesei* can efficiently degrade crystalline cellulose to glucose. The enzymes that hydrolyze the cellulose component can be divided into the following two types: endoglucanases (EC 3.2.1.4) which hydrolyze the internal bonds in the cellulose chains; and cellobiohydrolases (exoglucanases: EC 3.2.1.91) which hydrolyze from the chain ends and produce predominantly cellobiose [1]. The two cellolbiohydrolases secreted by *T. reesei* appear to be complementary in some respects. They exhibit synergy and have been shown to act at different ends of the cellulose chain; Cel7A acts at the reducing end, and Cel6A acts at the nonreducing end [2] (in this paper the nomenclature of Henrissat *et al.* [3] is used; Cel7A is the same as CBH I, and Cel6A is the same as CBH II). A good understanding of the adsorption phenomena concerning cellulase components may provide some clues as to the true reaction mechanism and the synergism of the cellulase complex. Recently some important observations on the adsorption and synergism of cellulase components have been reported [4,5]. The Papain digestion of both cellobiohydrolase I (Ce17A) and cellobiohydrolase II (Ce16A) from *T. reesei* results in the cleavage of amino acids from their C-terminal and N-terminal ends, respectively, which include the cellulose binding domains (CBDs) and most of the hinge region [6]. Limited proteolysis with papain has revealed a common structural organization for one group of cellulase from both fungal and bacterial origin [7,8]. In addition to an active site, which is located in the main part of the enzyme (the core), these enzymes have a short extra binding domain connected to the core via a flexible arm [9,10]. This organization improves the binding to and, therefore, the hydrolysis of crystalline cellulose [11]. A synthetic analogue has been shown to retain the adsorptive properties of the natural binding domain of *T. reesei* cellobiohydrolase I (Ce17A) and its three-dimensional structure has been determined using two-dimensional NMR [9,12]. A synergism exists between purified cellobiohydrolases (CBH) and endoglucanases (EG), as well as between Ce17A and Ce16A, however the conditions under which it may be important in an actual process of enzymatic cellulose hydrolysis are still unknown [13]. Such knowledge could allow cellulases to be used with greater efficiency. Although several studies have addressed the question of the adsorption of cellulase on cellulose using mathematical and practical cellulase on cellulose approaches, few data exist giving the equilibrium dissociation constants (K_d) and values for the maximum amounts of enzyme (N) that can be adsorbed onto a given quantity of cellulose for purified cellulase components [14,15].

*Corresponding author
Tel: +82-43-261-2281 Fax: +82-43-267-4232
e-mail: chem131@trut.chungbuk.ar.kr

The current authors previously described the adsorption kinetics of exoglucanase in combination with endoglucanases from *T. reesei* on microcrystalline cellulose and their influence on synergistic degradation [8]. It was shown that the synergistic degradation of microcrystalline cellulose is dependent on both the randomness of the endoglucanases and the tightness and affinity of adsorption.

The present work investigated the adsorption characteristics of Ce17A and Ce16A, and attempted to elucidate the mode of action of cellobiohydrolases and verify their synergistic effect.

MATERIALS AND METHODS

Enzymes

The commercial *T. reesei* cellulase preparation was a generous gifts from Novo Industries (Bagsvaerd, Denmark). The Ce17A and Ce16A were isolated from commercial cellulase derived from the fungus *T. reesei* based on a series of chromatography procedures involving Bio-Gel P 10 (Bio-Rad Laboratories, Richmond, USA), DEAE-Sepharose CL-6B, and SP-Sephadex C 50 (Pharmacia Fine Chemicals, Uppsala, Sweden) [16].

Enzyme Assay

The enzyme activities towards Avicel PH 101, H_3PO_4-swollen cellulose and CM-cellulose were measured, and recorded as international units (IU), in which one unit of activity is defined as the amount of enzyme required to liberate 1 μmol of reducing sugar per minute. The reaction mixture for the Avicelase activity was consisted of 1.6 mL of a 1% Avicel (w/v) suspension in a 0.01 M sodium acetate buffer (pH 4.8) and 0.4 mL enzyme solution. The incubation took place in a shaking incubator at 30°C for 20 h. After centrifugation, 1 mL of the supernatant was used to determine the reducing sugar using the DNS method. The CMCase activity was measured in a mixture containing 0.9 mL of a 1% CMC solution (w/v) in a 0.01 M sodium acetate buffer (pH 4.8) and 0.1 mL enzyme solution. After incubation at 40°C for 10 min, the reaction was stopped and the reducing sugar determined using the DNS method. The activity toward H_3PO_4-swollen cellulose was determined by placing 0.9 mL suspension of 1% (w/v) H_3PO_4-swollen cellulose in 0.01 M sodium acetate buffer, pH 4.8 and 0.1 mL of enzyme solution in a test tube. The mixture was incubated at 40°C for 2 h. After centrifugation 0.5 mL of supernatant was used to determine the reducing sugar by DNS method [17], with glucose as the standard.

Adsorption Studies

Avicel pH 101 was used as the cellulose adsorbent. A 50 mg cellulose sample was suspended in 1.0 mL of a 0.05 M sodium acetate buffer, pH 4.8, and preincubated at 5°C for 60 min. After preincubation, 4.0 mL of 0.05-2.0 mg/mL of the enzyme were added. The cellulases employed were Ce17A, Ce16A, and their mixtures (molar ratio, 1:1). The reaction mixture was subjected to reciprocal shaking at 120 strokes/min for 30 min, and then centrifuged for 5 min at 5,000 rev/min. The amount of enzyme in the supernatant was determined using fast protein liquid chromatography (FPLC) on the free concentrate of Ce17A or/and Ce16A present. Then, based on the free enzyme and residual substrate concentrations, the amount of bound enzyme was calculated using the initial enzyme concentration and expressed as the enzyme bound per gram of residual substrate.

Quantification of Proteins by FPLC

The amounts of free proteins in the supernatant from the adsorption experiments were quantitatively determined by means of anion-exchange chromatography on a Mono Q column in an automated FPLC system (Pharmacia). The method described here is a modification of the method suggested by Ellouz *et al.* [18]. A 10-30 μL sample was injected into a SuperLoop where it was diluted up to 6 mL with a start buffer (20 mM triethanolamine-HCl, pH 7.6). The diluted sample was then applied to the anion-exchange column and eluted using a 12 mL gradient (end buffer: 0.05 M NaCl in 20 mM triethanolamine-HCl, pH 7.6) at a 1 mL/min flow rate. The ultraviolet (UV) absorbance of the eluent was followed at 280 nm. Calibration curves based on the peak areas and peak heights were used to determine the amount of the enzyme(s) in the injected sample within a range of 4 pmol-1 nmol of enzyme in a 10-30 μL sample. It was found that FPLC was an accurate and reproducible method for quantify the enzyme adsorption of Avicel. Using ion-exchange chromatography in an automated FPLC system, Ce17A and Ce16A could both be analyzed in mixtures. Under the conditions used, Ce17A and Ce16A were completely separated and exhibited peaks with little or no tailing. The method was very reproducible and the standard deviation calculated from three to five injections was normally within a range of 3-6%.

Langmuir Model for Two Binding Site

Although a Langmuir isotherm has often been used to describe the adsorption of pure (or partly purified) cellulases to cellulose [19,20], the model in this section describes how the amount of the adsorbed substance (enzyme) depends on the concentration of the substance in the solution. Using a the Langmuir adsorption isotherm, the adsorption of cellulase can be described as follows:

$$[A] = n[E] / (K_d + [E]) \quad (1)$$

where n (mg/g) is the number of binding sites per gram of substrate and K_d (mg/L) is the dissociation constant

of the adsorbent-adsorbate complex. [*A*] (mg/g) is the amount of bound enzyme per unit weight of substrate and [*E*] (mg/L) is the free enzyme concentration in the solution. In addition to the K_d value, the affinity of the adsorbate to the adsorbent can also be characterized, beside the K_d value, based on the distribution coefficient equal to the ratio of the bound and the free substance [*A*]/[*E*] at an infinitely low concentration ([*E*] ≅ 0). The distribution coefficient is the initial slope of a Langmuir isotherm and can be calculated as:

$$\alpha = n / K_d \quad (2)$$

where α has the dimension L/g. The model can also be extended to assume the adsorption of more than one kind of binding site [21]. The Langmuir model for the two-binding-site adsorption of an enzyme can be described as:

$$[A] = n_1[E] / (K_{d1} + [E]) + n_2[E] / (K_{d2} + [E]) \quad (3)$$

where n_1 and n_2 (mg/g) are the maximum number of available sites per gram of substrate. K_{d1} and K_{d2} (mg/g) are the dissociation constants for the two sites. Although the underlying assumptions for a Langmuir isotherm (uniform binding sites and no interaction between the adsorbing molecules) are certainly not valid for the adsorption of cellulases to cellulose, this model was found to be useful to describe the experimental adsorption isotherms of (pure) cellulases. Plus it was flexible enough to follow the experimental data at both a low and high saturation of the substrate and gave two meaningful parameters to characterize the binding: the total number of available binding sites. *n* (mg/g),

$$n = n_1 + n_2 \quad (4)$$

and the overall distribution coefficient,

$$\alpha = n_1 / K_{d_1} + n_2 / K_{d_2} \quad (5)$$

For cellulases, where the adsorption is not (completely) reversible (thus no physical meaning can be associated with the K_d values), the distribution coefficient can be used to describe the affinity of the adsorbate to the adsorbent instead of K_d.

RESULTS AND DISCUSSION

The purified Ce17A and Ce16A showed a single band on an SDS/PAGE (Fig. 1). The average molecular weights determined by SDS-polyacrylamide gel electrophoresis analysis were 65,000 and 53,000 in Da for Ce17A and Ce16A respectively. The activities of Ce17A and Ce16A purified from *T. reesei* are summarized in Table 1. The specific activities of Ce17A and Ce16A had high H_3PO_4-swollen cellulose hydrolyzing activity and a relatively lower action toward Avicel, and negligible CM-cellulose activity. The activities of Ce16A for CM-cellulose and H_3PO_4-swollen cellulose were higher than that of Ce17A. While the activity of Ce16A for Avicel was slightly lower than that of Ce17A. The main hydrolysis product of the cellulose when using the two enzymes was cellobiose, thereby indicating that the two enzymes were indeed Ce17A and Ce16A (result not shown). The adsorption parameter was determined using the Langmuir adsorption equation in order to accomplish a quantitative comparison of the adsorption characteristics of cellobiohydrolase (Fig. 2). At most concentrations, Ce17A was adsorbed to a considerably higher extent than Ce16A. When Ce17A and Ce16A were studied individually and together, yet at a low enzyme/substrate ratio (E/S < 12 mg/g), the difference between them was very small. However, at high concentrations, a reduction in adsorption was observed in presence of the other enzyme; the adsorption of Ce16A

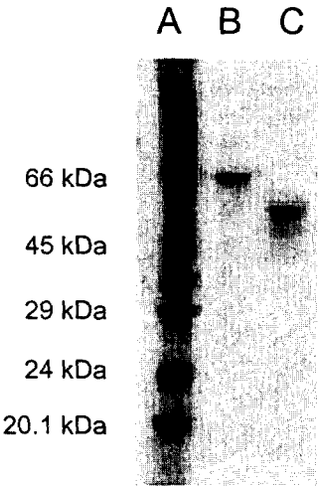

Fig. 1. SDS-polyacrylamide gel electrophoresis of Ce17A and Ce16A purified from commercial cellulase derived from *T. reesei*. The gel was 10% in a polyacrylamide concentration. The proteins were stained with Coomassie Brilliant Blue. The Marker proteins were bovine serum albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), and trypsinogen (24 kDa). (A) Marker, (B) Ce17A, (C) Ce16A.

Table 1. Specific activity of Ce17A and Ce16A proteins purified from *T. reesei*. The specific activities were measured at 30°C and pH 4.8 toward Avicel, and at 40°C, and pH 5.0 towards CM-cellulose and H_3PO_4-swollen cellulose

Enzyme	Specific activity (IU/mg)		
	CM-cellulose[a]	Avicel	H_3PO_4-swollen cellulose[b]
Ce17A	0.01	0.07	8.01
Ce16A	0.02	0.06	13.20

[a] Carboxymethylcellulose
[b] Amorphous cellulose regenerated with phosphoric acid

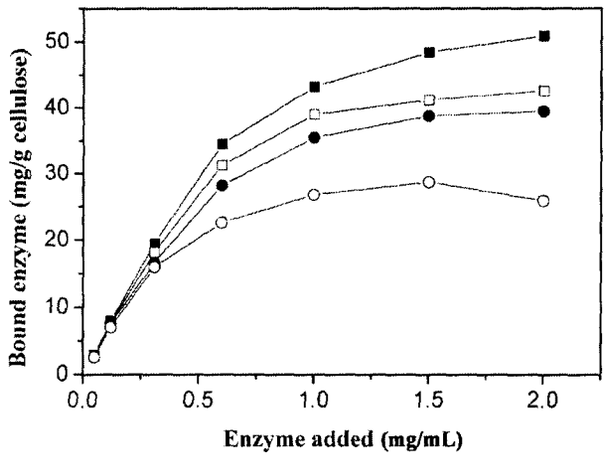

Fig. 2. Adsorption isotherms of Ce17A and Ce16A after 30 min incubation at 5°C. The enzymes were studied individually in mixtures with a 1:1 molar ratio. The enzyme/substrate ratio was varied within a range of 1-40 mg/g. ■ Ce17A; □, Ce16A; ●, Ce17A in the presence of Ce16A; O, Ce16A in the presence of Ce17A.

was more strongly influenced by the presence of Ce17A than vice versa in contrast. At a low saturation, the effect of the other enzyme was negligible. The decrease in adsorption when the two cellobiohydrolases were studied together was a result of competition for binding sites on the cellulose. Based on calculating the total enzyme adsorption and then comparing it with the isotherms for the single enzymes, besides competition for common binding sites, it was clear that adsorption to sites that were specific for Ce17A and Ce16A also contributed to the total adsorption.

A plot of the ratio of the bound-to-free-protein against the bound-protein at equilibrium resulted in the determination of the dissociation constant, K_d (negative reciprocal of the slope), and number of binding sites, n, that bound to the substrate Avicel PH 101 (Fig. 3). This data is given in Table 2. The scatchard plots for the binding of all the cellulase components studied were nonlinear, which can be explained due to the existence of amorphous (high K_d values) and crystalline (low K_d values) regions in the substrate [22]. Purified Ce17A had higher K_d values for the crystalline regions of Avicel PH 101 than Ce16A, which can explain previous observations that the binding of Ce16A to cellulose is stronger than that of Ce17A, based on the finding that Ce16A is much more difficult to elute from a column of cellulose than Ce17A [21]. The non-linear Scatchard plots (Fig. 3) showed that the adsorption was not a true Langmuir type, but rather involved multiple binding site classes and negative cooperativity. Using a two-site model, apparent capacities and dissociation constants were derived from the adsorption isotherms through a non-linear regression analysis (Table 2). Since the actual number of binding site classes was unknown, the two sites were only considered as virtual sites and their derived constants as apparent. In all three cases the binding parameters obtained matched well with the experimental data [23]. The distribution coefficients (Table 2) were higher for Ce16A than for Ce17A, indicating a stronger binding of Ce16A. In contrast, the binding capacities, were higher for Ce17A. The higher the extent of degradation, the lower the dissociation constant and binding capacity. This supports the hypothesis: the wedge-shaped tail of Ce17A plays a more active role in facilitating the release of a single cellulose chain from a crystalline surface [24]. Accordingly, the fact that the maximal synergism for Ce17A in combination with Ce16A was produced at a high ratio may have been related to the endo type of attack and active role of Ce17A in facilitating the release of single cellulose chains. Thus a lower proportion of Ce17A may provide a sufficient number of chain ends for Ce16A and a Ce17A-Ce16A complex to act on [19]. As such, these binding parameters result in a higher amount of bound Ce17A compared to Ce16A. The lower binding capacity observed for Ce16A was in accordance with the observations made using electron microscopy, where Ce16A bound preferably to cellulose crystal ends and Ce17A bound over the whole length of the crystal [24], yet conflicted with the data of Tomme *et al.* [16], who found the binding capacity for Ce16A to be similar to

Table 2. Apparent dissociation constants and binding capacities for adsorption to Avicel PH-101 of *T. reesei* cellobiohydrolases when studied alone and in equimolar mixtures. The values are derived from adsorption isotherms based on a non-linear regression analysis using the two-site Langmuir model (Eq. [3-5])

Protein	Dissociation constants (mg/L)		Binding capacities (mg/g-Avicel)			Distribution coefficient (L/g)		
	High[a] (K_{d1})	Low[b] (K_{d2})	High (n_1)	Low (n_2)	Total	High (α_1)	Low (α_2)	Sum
Ce17A alone	2.63	10.03	24.39	45.58	69.97	9.27	4.55	13.82
Ce16A alone	0.72	6.31	8.44	21.96	30.4	11.81	3.48	15.29
Ce17A in presence of Ce16A	1.53	6.97	13.50	23.76	37.26	8.84	3.41	12.25
Ce16A in presence of Ce17A	0.30	3.61	5.15	11.54	16.69	17.19	3.19	20.38

[a] High affinity site, [b] Low affinity site

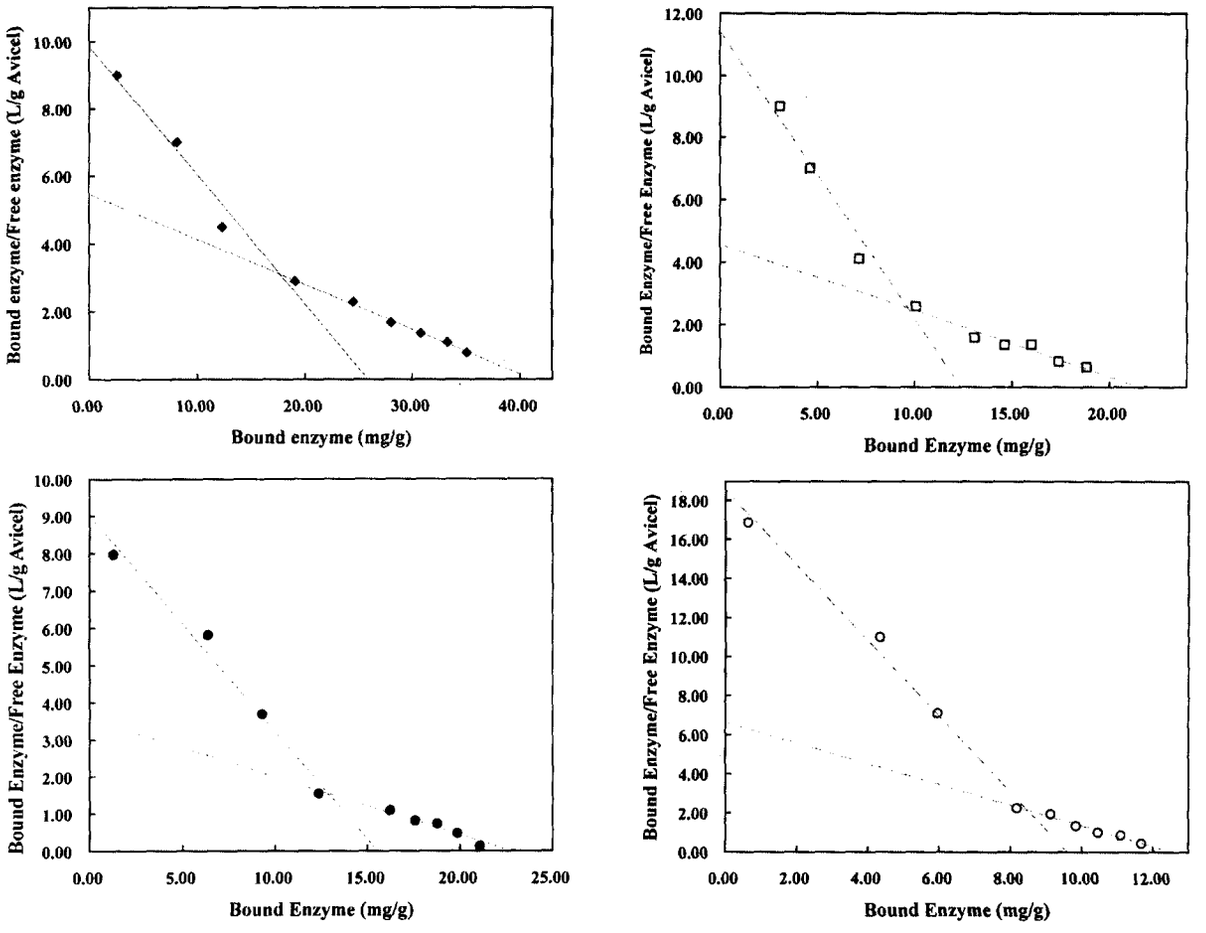

Fig. 3. Scatchard analysis for equilibrium binding data of *T. reesei* purified Ce17A and Ce16A to Avicel PH 101. The results were the means of triplicate determinations. The lines show the best fit obtained from a two-site model using a non-linear regression analysis. The individual functions are for high (high K_d values) and low affinity sites (low K_d values). ■, Ce17A; □, Ce16A; ●, Ce17A in the presence of Ce16A; O, Ce16A in the presence of Ce17A.

that for Ce17A at 64 and 70 mg/g, respectively. Negative cooperativity and/or the existence of multiple binding site classes can lead to a concave Scatchard plot. Occasionally, non-linear plots of cellulase-cellulose adsorption can be interpreted as strictly biphasic, representing binding to crystalline and amorphous regions, however, there was no direct evidence for this in the current plot. The two-site Langmuir model was previously used by Woodward *et al.* [22] and Stahlberg *et al.* [20] to describe the adsorption of pure cellulases to Avicel. In this study, the binding parameters for Ce17A (Table 2) were similar to those obtained by Stahlberg *et al.* [20], despite differences in the experimental methodology used for each study.

The adsorption parameters with the enzyme system of *T. reesei* were strongly dependent on the competitive adsorption of the individual components. A decrease in the adsorption was observed when the two cellobiohydrolases were studied together due to competition for binding sites on the cellulose. Ce17A competed more efficiently for the binding sites than Ce16A. Competition for binding sites was the dominating factor when the two enzymes were acting together, however, adsorption to sites specific for Ce17A and Ce16A, also, contributed to the total adsorption.

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