Effect of Metal Ions on the Degradation and Adsorption of Two Cellobiohydrolases on Microcrystalline Cellulose

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To test the metal ion effect, hydrolysis experiments for two cellobiohydrolases (CBH I and CBH II) from *Trichoderma reesei* have been carried out in the presence of 10 mM metal ions, such as Cu^{++}, Mn^{++}, Ca^{++}, Hg^{++}, Ba^{++}, Pb^{++}, and Cd^{++}. The addition of Mn^{++}, Ba^{++}, and Ca^{++} (10 mM) during the hydrolysis of Avicel PH 101 caused an increase in the total reducing sugar (TRS) for CBH I by 142, 135, and 114 percent, respectively. Those for CBH II increased by 177, 175, and 115 percent, respectively. The Mn^{++} was the most stimulatory metal ion, whereas Hg^{++} was the most inhibitory metal ion. The adsorption experiments were performed to investigate how the influence of Mn^{++} and Hg^{++} on the hydrolysis is related to the adsorption of cellobiohydrolases on cellulose. The increase in TRS during hydrolysis by adding Mn^{++} caused an increase in adsorption affinity (K_{ad}) and tightness (ΔH_a). While, the decrease of TRS during hydrolysis by adding Hg^{++} caused a decrease in the adsorption affinity (K_{ad}) and tightness (ΔH_a). These results indicate the changes in the tightness and affinity of adsorption by adding metal ions play a crucial role in the degradation of the microcrystalline cellulose.

Keywords : Cellobiohydrolases, Metal ion, Adsorption, Degradation, Kinetics.

Introduction

Cellulosic materials are annually renewable resources. The utilization of the monomeric sugars stored in cellulosic substrates for the production of industrial chemicals and microbial protein requires prior hydrolysis of the polysaccharides by acid or enzymatic treatment. Enzymatic hydrolysis, one of the most effective methods to degradate cellulose, has been studied closely during the last few decades.[1,2] However, how cellulases in a multi-enzyme system act on the complex surface of the cellulose for its degradation remains a mystery.

Today, there are relatively few studies on the interaction between metal ions and cellulase. Some researches have explored to the effects of metal ions as inhibitory agents. Kanamoto *et al.*[3] reported that Cu^{++} and Hg^{++} are generally inhibitory. On the other hand, $Ca^{++}$and Mg^{++} have been found to be either stimulatory or at least required for stability, though in relatively few cases.[4] Also, Johnson *et al.*[5] reported that the cellulase activity from *c. thermocellum* depends on the presence of Ca^{++} and a thiol reducing agent. But, the relationships among cellulase components, metal ions, and cellulose are not sufficiently understood. Nevertheless, these observations may provide clues to describe exactly how cellulase components act to cellulose.

Recently, there has been considerable interest in utilizing adsorption to obtain a better understanding of the mechanism of enzymatic degradation. A good understanding of the adsorption phenomena of cellulase components may provide some clues to the true reaction mechanism. Several attempts to specifically quantify the individual component in cellulase mixtures during adsorption experiments are described.[6,7,8]

We have previously described the adsorption kinetics of exoglucanase (Exo II) in combination with endoglucanase (endo I, II, III, and IV) from *Trichoderma viride* on microcrystalline cellulose and its influence on synergistic degradation. The study shows that synergistic degradation of microcrystalline cellulose is dependent on the randomness of the endoglucanase and the tightness and affinity of adsorption.[9] Also, we reported recently that the increase of affinity (K_{ad}) and tightness (ΔH_a) for the adsorption of CBH I combined with CBH II is in parallel with their maximum synergistic degradation.[10,11]

In the present work, we investigate the degradation and adsorption characteristics on microcrystalline cellulose for CBH I and II in the presence of divalent metal ions. Adsorption experiments were performed to investigate how the influence of the metal ions on the hydrolysis of cellulose is related with the adsorption of CBH I and II.

Experimental Section

Enzymes. CBH I and II were isolated from commercial *Trichoderma reesei* cellulase preparation (EC 3.2.1.4, Fluka) by a series of chromatography procedures involving Bio-Gel P 10 (Bio-Rad Laboratories, Richmand, USA), DEAE-Sepharose CL-6B and SP-Sephadex C 50 (Pharmacia Fine Chemicals, Uppsala, Sweden) as described in reference[12]. As shown in Figure 1, the purified cellobiohydrolases showed a single band on SDS-polyacrylamide gel electrophoresis. The average molecular weights determined by SDS-polyacrylamide electrophoretic analysis were 65,000 and 53,000 in Da for CBH I and II, respectively. CBH I and

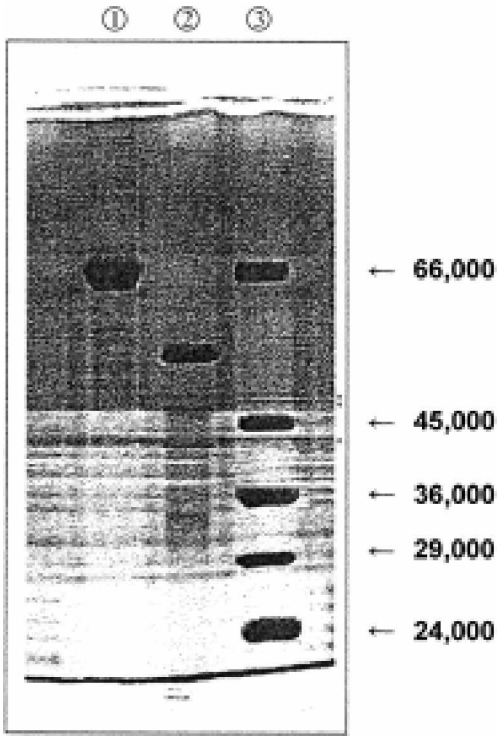

Figure 1. SDS-polyacrylamide gel electrophoresis of CBH I and II purified from commercial cellulase derived from *T. reesei*. The gel had a polyacrylamide concentration of 10%. ①: CBH I; ②: CBH II; ③: Marker proteins. The marker proteins were bovine serum albumin (Mr 66,000), egg albumin (Mr 45,000), glyceraldehyde-3-phosphate dehydrogenase (Mr 36,000), carbonic anhydrase (Mr 29,000) and trypsinogen (Mr 24,000).

II had high specific activity toward Avicel and very low activity toward CM-cellulose compared with endoglucanases. CBH II had lower specific activity compared with CBH I. The hydrolysis products of cellulose by the two enzymes were mainly cellobiose, indicating the two enzymes were indeed cellobiohydrolases.

Enzymatic Hydrolysis. Avicel PH 101 was used as the substrate for the hydrolysis. A 50 mg sample of Avicel PH 101 was accurately weighed, then immersed in 5 mL enzyme buffer solution at pH 4.8 containing 10 mM of the metal ions of $CuCl_2$, $MnCl_2$, $HgCl_2$, $CaCl_2$, $BaCl_2$, $PbCl_2$, and $CdCl_2$ then incubated at 50 °C with shaking at 120 strokes min^{-1}. The final enzyme concentration was 0.2 mg/mL. The reaction was stopped after 24 h by boiling for 10 min. The amount of reducing sugar released was estimated by the dinitrosalicylic acid (DNS) method, using glucose as a standard.[13]

Adsorption Studies. Avicel PH 101 was used as the cellulose adsorbent. A 50 mg cellulose sample was suspended in 1.0 mL of 0.05 M sodium acetate buffer, pH 4.8, and preincubated at a given temperature range of 15-30 °C for 60 min. After preincubation, 4.0 mL of 0.1-1.5 mg/mL enzyme preparation was added. The reaction mixture was subjected to reciprocal shaking at 120 strokes min^{-1} for 30 min and then centrifuged for 5 min at 5,000 rev min^{-1}. Adsorption experiments were carried out in the presence of 10 mM metal ions (Mn^{++} and Hg^{++}). The amount of enzyme in the supernatant was determined by the Lowry method,[14] using bovine serum albumin as the standard.

Table 1. Effect of metal ions on hydrolysis of cellulose

Metal Ions	CBH I		CBH II	
	TRS[a] (mg/mL)	Relative TRS (%)	TRS (mg/mL)	Relative TRS (%)
Buffer	0.159	100(control)	0.100	100(control)
Mn^{++}	0.226	142	0.178	177
Cd^{++}	0.137	86.2	0.090	89.5
Cu^{++}	0.013	8.2	0.011	11.0
Ba^{++}	0.215	135	0.175	175
Ca^{++}	0.182	114	0.115	115
Hg^{++}	0.002	1.1	0.003	3.0
Pb^{++}	0.009	5.7	0.014	14

[a]Total reducing sugar.

Results and Discussion

Effect of Metal Ions on Hydrolysis. To test the metal ion effect, hydrolysis experiments were carried out in the presence of 10 mM of the metal ions of $CuCl_2$, $MnCl_2$, $HgCl_2$, $CaCl_2$, $BaCl_2$, $PbCl_2$, and $CdCl_2$. The influence of the divalent cations on the hydrolysis for CBH I and CBH II is shown Table 1. The relative TRS on hydrolysis are given relative to the controls (set equal to 100 in Table 1) with no additives. The addition of Mn^{++}, Ba^{++}, and Ca^{++} during the hydrolysis of cellulose caused an increase in the total reducing sugar (TRS). This result means the degradation of cellulose was stimulated by these metal ions. On the other hand, a significant decrease of TRS during the hydrolysis was observed with Hg^{++}, Cu^{++}, and Pb^{++}. This indicates the degradation of cellulose was strongly inhibited by Hg^{++}, Cu^{++}, and Pb^{++}. The values of TRS for CBH II by Mn^{++} and Ba^{++} (177 and 175 percent to relative TRS, respectively) were higher than those of CBH I (142 and 135 percent relative TRS, respectively). This indicates CBH II is more sensitive than CBH I to these metal ions. Also, addition of Ca^{++} for CBH I and II improved the TRS slightly. The Mn^{++} was the most stimulatory metal ion, while Hg^{++} was the most inhibitory metal ion. These results for Mn^{++} and Hg^{++} led us to study its effects in greater detail.

Effect of Metal Ions on Adsorption. The adsorption experiments were performed to investigate how the influence of the metal ions on the hydrolysis of cellulose is related to the adsorption of cellobiohydrolases. The Mn^{++} metal ion obtained maximum TRS, and Hg^{++} metal ion obtained minimum TRS during hydrolysis of cellulose as adding agents for the adsorption experiments. Adsorption experiments were carried out in the absence and presence of $MnCl_2$ and $HgCl_2$ (10 mM). The adsorption kinetic results of cellobiohydrolases (CBH I and II) from *Trichoderma reesei* show that 60 min is needed to reach equilibrium. Therefore, this incubation time was chosen for the isotherm experiments. Also, the amount of adsorption after a 60 min contact

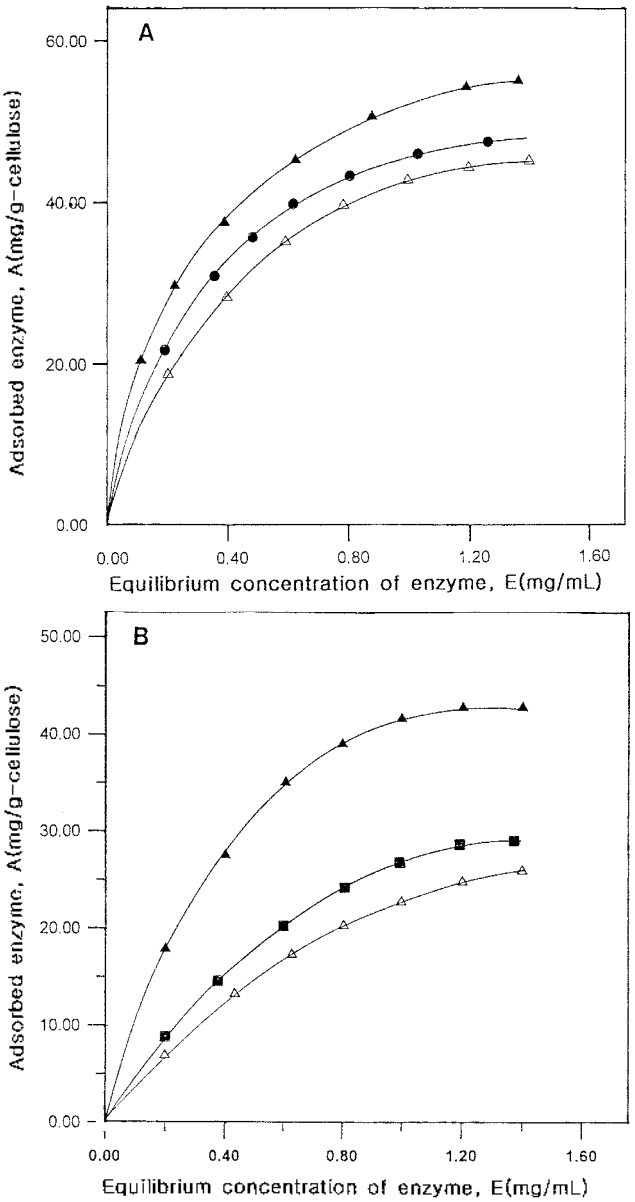

Figure 2. Adsorption isotherms of enzyme components in the presence of metal ions on Avicel 101 at 25 °C. (A): CBH I. (●) no metal ion; (▲) Mn^{++}; (△) Hg^{++}. (B): CBH II. (■) no metal ion; (▲) Mn^{++}; (△) Hg^{++}.

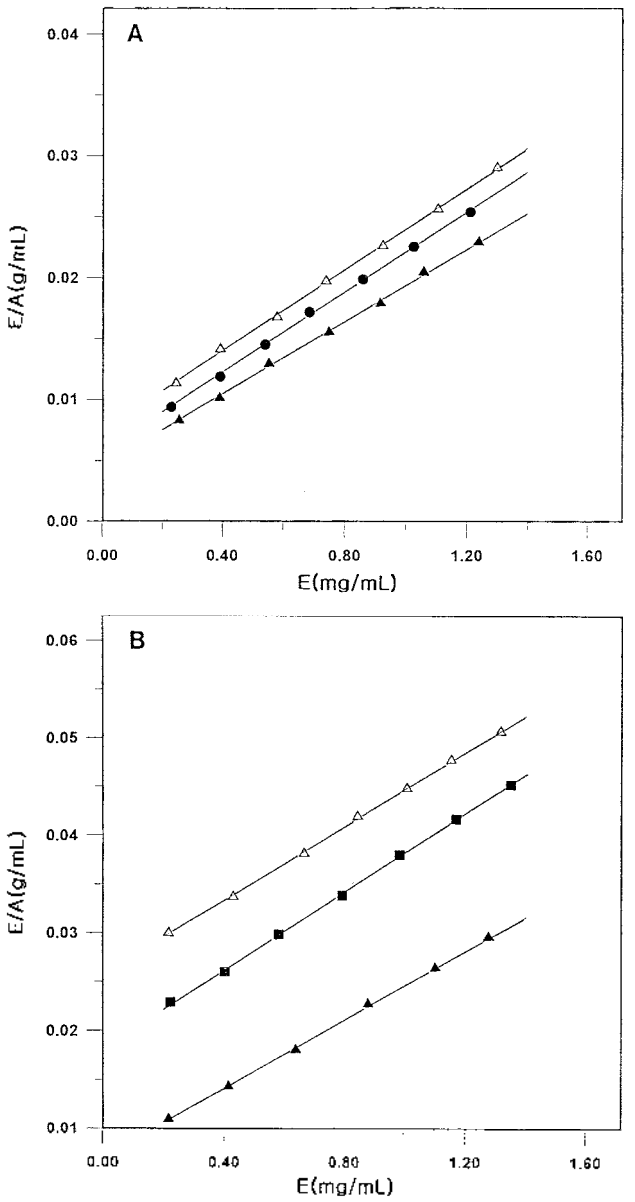

Figure 3. Langmuir plots for the adsorption isotherms of enzyme components in the presence of metal ions. (A): CBH I. (●) no metal ion; (▲) Mn^{++}; (△) Hg^{++}. (B): CBH II. (■) no metal ion; (▲) Mn^{++}; (△) Hg^{++}.

period was taken as the maximum adsorbed value.

The parameter values were determined using the Langmuir adsorption isotherm equation to obtain a quantitative analysis of the effect of the composition of cellulase components.

From the Langmuir adsorption isotherm, the adsorption of cellulase can be described as follows:

$$[A] = \frac{[A_{max}] \cdot K_{ad}[E]}{1 + K_{ad}[E]} \quad (1)$$

where A_{max} and K_{ad} are the maximum amount of enzyme adsorbed per unit weight of cellulose and the adsorption equilibrium constant, respectively. $[E]$ is the concentration of enzyme in the liquid phase at the adsorption equilibrium.

Figure 2 shows adsorption as a function of varying enzyme concentrations at 25 °C. The adsorption isotherm. equation (1), can be rearranged as follows:

$$\frac{[E]}{[A]} = \frac{1}{K_{ad} \cdot [A_{max}]} + \frac{1}{[A_{max}]} \cdot [E] \quad (2)$$

The adsorption equilibrium constant and amount of maximum adsorption were determined from the slope of plots of $[E]/[A]$ *vs.* $[E]$, using a least-square analysis. The plots of $[E]/[A]$ *vs.* $[E]$ gave fairly good straight lines. as shown in Figure 3. These results imply that the adsorption of CBH I and CBH II on cellulose follow the Langmuir isotherm, as also observed by other investigations.[15-17] Equation (2) is valid only if the adsorption site and adsorbate molecules are

Table 2. Langmuir parameters at various temperatures

Temp. (°C)	CBH I						CBH II					
	Buffer		Mn^{++}		Hg^{++}		Buffer		Mn^{++}		Hg^{++}	
	$^aA_{max}$ $\times 10^4$	$^bK_{ad}$ $\times 10^{-4}$	$A_{max}\times 10^4$	K_{ad} $\times 10^{-4}$	A_{max} $\times 10^4$	K_{ad} $\times 10^{-4}$	A_{max} $\times 10^4$	K_{ad} $\times 10^{-4}$	A_{max} $\times 10^4$	K_{ad} $\times 10^{-4}$	A_{max} $\times 10^4$	K_{ad} $\times 10^{-4}$
15	9.70	23.4	11.14	28.6	9.55	16.2	9.77	6.38	12.84	16.6	10.22	4.1
20	9.53	21.0	10.76	24.0	9.34	15.6	9.60	6.05	12.09	14.6	10.01	4.0
25	9.35	18.4	10.40	20.5	9.25	14.4	9.36	5.80	10.75	13.0	9.94	3.9
30	8.49	16.2	9.72	17.2	8.95	13.3	9.00	5.60	10.39	11.3	9.47	3.9

[a]Maximum adsorption amounts of cellulase (mol kg^{-1} cellulose). [b]K_{ad} = adsorption equilibrium constant (L mol^{-1}).

dependent and equivalent and is not applicable to multi-equilibrium systems comprising two different adsorbates. in general. Nevertheless. adsorption data for CBH I and CBH II are known to obey the Langmuir adsorption isotherm. Table 2 shows the Langmuir parameters for cellulase. which were estimated from the data in Figure 3. The value of K_{ad} and A_{max} decreased with increasing temperature. and this tendency was remarkable in the case of CBH I. The increase in K_{ad} and A_{max} at lower temperatures may be related to a reduction in translational energy of the enzyme. which could enhance the positioning of the enzyme for adsorption. or to a reduction in energy available for desorption. The fact that A_{max} and K_{ad} increased with decreasing temperature means. according to the Le Chatelier principle, that the adsorption of cellulase is driven by a decrease in enthalpy. which is an evolution of the heat of adsorption. The values of A_{max} in the presence of Mn^{++} for CBH I and II were larger than those in the absence of metal ion. The values of A_{max} in the presence of Hg^{++} for CBH I were smaller than those in the absence of metal ion. The values of A_{max} in the presence of Hg^{++} for CBH II were larger than those in the absence of metal ion. The amount of maximum adsorption by adding Hg^{++} was very large compared with very small TRS in hydrolysis. This means that the amount of TRS during hydrolysis is not dependent upon the value of A_{max}. The K_{ad} values of CBH I were larger than those of CBH II. The K_{ad} value is an intensive property of adsorption and is a measurement of adsorption affinity. Therefore, CBH I had a higher adsorption affinity for Avicel than CBH II.

The values of K_{ad} in the presence of Mn^{++} are larger than the values in its absence. This indicates that the adsorption affinity is increased by Mn^{++}. Whereas the values of K_{ad} in the presence of Hg^{++} are smaller than the values in its absence. This indicates that adsorption affinity is reduced by Hg^{++}. These results indicate that the metal ions affect the affinity of adsorption for CBH I and CBH II during the adsorption process. This means that metal ions can be related to the interaction between the binding domain of cellulase and the binding site of cellulose. The changes of K_{ad} between the presence and absence of Mn^{++} for CBH II were greater than those of CBH I. The value of TRS for CBH II by Mn^{++} (177 percent to relative TRS) was higher than that of CBH I (142 percent to relative TRS). This indicates that the improvement of K_{ad} by metal ion is related to the degradation of cellulose.

The thermodynamic parameters in the adsorption of

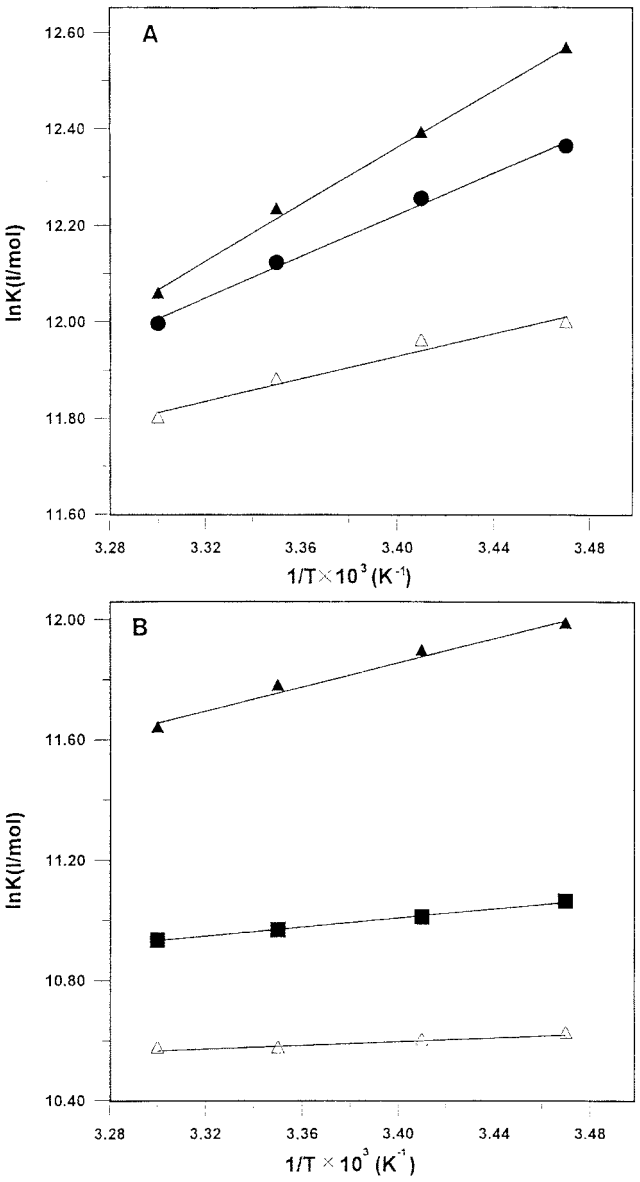

Figure 4. Temperature dependency of adsorption equilibrium constant (K_{ad}) of enzyme components on Avicel 101. (A): CBH I. (●) no metal ion; (▲) Mn^{++}; (△) Hg^{++}. (B): CBH II. (■) no metal ion; (▲) Mn^{++}; (△) Hg^{++}.

Table 3. Thermodynamic parameters in adsorption cellulase components at 25 °C

Thermodynamic Parameters	CBH I			CBH II		
	Buffer	Mn^{++}	Hg^{++}	Buffer	Mn^{++}	Hg^{++}
ΔH_a (kJ mol^{-1})	-18.0	-24.4	-9.7	-6.2	-17.0	-2.6

cellulase can be evaluated from the K_{ad} values. The change in enthalpy (ΔH_a), one of the thermodynamic parameters, was obtained from the van't Hoff equation:

$$d\ln K_{ad} = \left(\frac{-\Delta H_a}{R}\right) d\left(\frac{1}{T}\right) \quad (3)$$

where ΔH_a is the value of enthalpy of adsorption, and R is a gas constant. Equation 3 shows that a plot of $\ln K_{ad}$ against $1/T$ has a slope of $-\Delta H_a/R$ (Figure 4).

Table 3 shows the changes of enthalpy accompanying the adsorptions that were obtained with Equation (3). We found from the negative values of ΔH_a that the adsorption reaction was an exothermic and adsorption enthalpy-controlled reaction. Therefore, the adsorption of CBH's is driven by the decrease in enthalpy in the formation of the enzyme-cellulose complex. In the previous article, we reported that it was effective to describe the change of adsorption enthalpy as the tightness of the cellulase adsorption on cellulose.[9] The heat of adsorption of CBH I is -18.0 kJ mol^{-1}, much larger than that of CBH II (-6.2 kJ mol^{-1}). It can be concluded that CBH I shows stronger preferential adsorption than CBH II.

The values of negative ΔH_a in the presence of Mn^{++} are larger than the values in its absence. This indicates that the adsorption tightness is increased by Mn^{++}, whereas the values of negative ΔH_a in the presence of Hg^{++} are far smaller than those in its absence. This indicates that the adsorption tightness is reduced by Hg^{++}. These results indicate that the metal ions would affect the tightness of adsorption for CBH I and CBH II during the adsorption process.

The K_{ad} and negative ΔH_a values for CBH I are much larger than those of CBH II. This indicates that the CBH II does not affect the adsorption of CBH I significantly. Also, these findings support the view that the specific binding site exists for CBH I and II, respectively.[18] Accordingly, the conclusion may be drawn that the difference of adsorption affinity and tightness for CBH I and II in the presence of metal ions may be caused by the difference of metal ion interactions with these enzymes on a specific binding site.

The increase of TRS during hydrolysis by adding Mn^{++} caused an increase of the adsorption affinity (K_{ad}) and tightness (ΔH_a), whereas the decrease of TRS on hydrolysis by adding Hg^{++} caused a decrease of adsorption affinity (K_{ad}) and tightness (ΔH_a). This means the amounts of TRS on hydrolysis by metal ions is directly correlated with the adsorption affinity (K_{ad}) and tightness (ΔH_a). A strong affinity and tightness of the enzyme would disrupt the hydrogen bonding networks in the crystal lattice, ultimately collapsing the ordered structure of cellulose particles and preventing their adhesion. These results indicate that the changes of the tightness and affinity of adsorption by adding metal ions play a crucial role in the degradation of the microcrystalline cellulose.

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