

## THE ROLE OF CELLULOSE BINDING DOMAINS IN THE ADSORPTION OF CELLULASES ONTO FIBERS AND ITS EFFECT ON THE ENZYMATIC BEATING OF BLEACHED KRAFT PULP

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The adsorption of cellulases onto fibers may be one of the most important factors affecting the enzymatic reaction between cellulases and fibers. This study investigated the adsorption kinetics involved, using isothermal adsorption equations. Cellulose binding domains (CBDs) were isolated from a commercial cellulase, and their role in the adsorption and enzymatic reaction was evaluated. Approximately 13% to 24% of the refining energy was saved after northern bleached softwood kraft pulp samples were pretreated with full cellulase, CBDs, or cellulase lacking CBDs under optimal conditions. The absence of CBDs in cellulase resulted in less effective enzyme adsorption and hydrolysis of the fibers. These data suggest that pretreatment of northern bleached softwood kraft pulp with CBDs may not only improve the beating degree of the pulp and reduce refining energy consumption but also improve the tensile index of the handsheet. Analysis of the degree of cellulose crystallinity and fiber surface morphology by X-ray diffraction and scanning electron microscopy revealed that the CBDs in cellulase help modify the crystalline area and facilitate the enzymatic degradation of cellulose. The adsorption parameters of the cellulases calculated from isothermal adsorption experiments confirmed the role of CBDs in the adsorption of cellulases onto fibers.

*Keywords: Cellulases; Isothermal adsorption equation; Cellulose binding domains (CBDs); Fiber modification*

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### INTRODUCTION

Energy conservation has become a priority for the paper industry due to the diminishing availability of fossil fuel and the high cost of energy (Liu and Hu 2011). Refining/beating, one of the essential steps in the papermaking process, requires substantial energy for the development of desired pulp properties. For example, greaseproof paper is a high-density paper produced by extensive refining of chemical pulps that involves considerable electrical energy consumption. Consequently, alternative approaches that minimize such high energy consumption are being sought. Spurred by recent advances in technology, one of the promising steps in the production of good-quality paper with less refining energy is the introduction of “enzymatic refining”. Cellulase and hemicellulase have been used in numerous industrial applications, including improving the beatability/refinability of pulps (Bhardwaj et al. 2010). Mechanical refining may reach a target beating degree more easily via partial hydrolysis

or modification of fibers with cellulase and hemicellulase, which provide gentler targeted refining.

The cellulase system, which hydrolyzes cellulose chains with a synergic effect, contains three types of enzymes: cellobiohydrolases, endoglucanases, and  $\beta$ -D-glucosidases. Cellobiohydrolases are exo-glucanases that hydrolyze cellulose chains from the ends and release cellobiose; endoglucanases randomly attack the amorphous regions of the cellulose substrate, yielding a high degree of polymerization oligomers; and  $\beta$ -D-glucosidases further break down dimers into glucose. Most cellulases have a general structure that comprises three parts: the catalytic domain, the cellulose binding domain (CBD), and the linker region. CBDs are supposedly responsible for the binding of enzymes onto cellulose, and they improve the binding as well as facilitate the activity of the catalytic domain in insoluble substrates, but not that in soluble substrates. Research has shown that the adsorption of cellulases and their hydrolytic activity are reduced if CBDs have been removed from the protein (Markus 1997). Despite these data, the significance of CBDs in the hydrolysis of cellulose remains unclear. Some studies have demonstrated that the CBDs of exo-glucanases are able to disrupt crystalline cellulose, thereby facilitating the enzymatic degradation of cellulose (Chunhui et al. 1997). The binding of CBDs to cellulose under a wider range of environmental conditions and without the need for chemical reactions makes them attractive molecules for the design of a new class of environment-friendly paper-modifying agents (Levy et al. 2000; Shoseyov et al. 2003).

Generally, the hydrolysis of cellulose by cellulases consists of two steps. As shown in the enzymatic reaction below (Eq. (1)), substrates ( $S$ ) combine with enzymes ( $E$ ) and form complexes ( $ES$ ). The complexes are subsequently decomposed, releasing the products ( $P$ ) and enzymes. Each elementary reaction in Eq. (1) includes  $k_1$  and  $k_{-1}$ , representing the forward and reverse reaction rate constants of the ES, respectively (Fuchu 2003).



The enzymatic hydrolysis of cellulose fibers with cellulase is a heterogeneous reaction process. Cellulose molecules bind together to form a strong structure, rendering the adsorption of cellulases onto the surface or their spread into the inner area of the fibers, which is critical to the modification of cellulose fibers.

Liu and Hu (2011) reported that pretreatment of pulp with cellulases containing CBDs results in greater improvement of the beating degree of the pulp and that such cellulases potentially conserve refining energy compared with cellulases lacking CBDs. However, their comparison of these two types of cellulases does not offer evidence to support the beneficial role of CBDs in cellulase adsorption during pulp pretreatment and the separation of CBDs from full cellulase as well as its application in pulp pretreatment.

The present study used papain to digest cellulase B in preparing cellulase with and without CBDs, as previously described (Lemos et al. 2000). These products were used to evaluate the enzymatic beatability of northern bleached softwood kraft (NBKP) pulp and to understand the role of CBDs in cellulases. The parameters of cellulase

adsorption onto fibers were investigated through isothermal adsorption experiments. Moreover, the degree of fiber crystallinity and fiber surface morphology were analyzed.

## EXPERIMENTAL

### Materials

The NBKP pulp used in the present study was supplied by Huatai Paper Group (Shandong Province, China). Cellulase B from *Trichoderma* and containing CBDs (Table 1) was provided by an enzyme corporation based in the United States.

**Table 1.** Characterization of Cellulase Preparations

Enzymes	Optimum temp. (°C)	Optimum pH	protein content (mg/mL)	Activity (U/mL)		
				FPA	EG	CBH
Cellulase B	45	4-6	15.21	956	1300	120
CBD	ND	ND	3.6	5.8	8.7	2.1
No-CBD	ND	ND	10.2	687	1210	52

Note: FPA indicates filter paper activity; EG, endoglucanase activity; CBH, cellobiohydrolase activity; ND, not detected.

The endoglucanase, filter paper, and cellobiohydrolase activities of the cellulases were measured using carboxymethylcellulose (1% w/v) as soluble substrate and filter paper (Whatman no. 1, 50 mg) as well as absorbent cotton (50 mg) as insoluble substrates. The amount of sugar released was measured using a dinitrosalicylic acid assay with glucose as the standard based on the industry-accepted QB 2583-2003 (CN) and the method applied by Ghose et al. (1987). Protein concentrations were determined by the Bradford (1976) method using a Bradford Protein Assay Kit (Beyotime, China). The calibration curve was made with bovine serum albumin as the standard.

### Methods

#### *Calculation of protein adsorption*

Measuring the amount of protein that can be adsorbed onto the fibers is crucial to better understanding the adsorption of enzymes onto fibers and its effects on the modification of fibers. According to Shen (2000), when the initial enzyme concentration  $[P_0]$  is constant and the consistency of fiber substrate is much higher than that of the enzyme, the relationship between the equilibrium adsorption amount of cellulases and the consistency of fibers can be expressed as follows,

$$\frac{[ES]}{[P_0]} = \frac{\alpha[S_0]}{(K_1 + S_0)} \quad (2)$$

where  $[ES]$  is the equilibrium adsorption amount (grams per liter) of the cellulases,  $[P_0]$  is the total amount of water-soluble protein added at the beginning of the adsorption experiments (grams per liter) that consists of the available enzyme protein that can be adsorbed onto the fiber surface and the inactive protein,  $\alpha$  is the fraction of adsorbable

enzyme protein accounting for the total water-soluble protein,  $[S_0]$  is the consistency of the fiber substrate for cellulase adsorption (grams per liter), and  $K_1$  is the semi-saturated adsorption equilibrium constant of the fiber substrate (grams of fiber per liter).

The isothermal adsorption equation that established the relationship between  $[ES]$  and the free enzyme consistency in the liquid phantom  $[P]$  can be shown as Eq. (3) when the consistency of the fibers  $[S_0]$  is constant,

$$\frac{[ES]}{[S_0]} = m \cdot \frac{\{[P] - [P_0](1 - \alpha)\}}{\{K_2 + [P] - [P_0](1 - \alpha)\}} \quad (3)$$

where  $m$  is the maximum amount of enzyme that can be adsorbed onto each gram of fiber and  $K_2$  is the semi-saturated adsorption equilibrium constant of cellulases (grams of protein per liter). The concentration of fibers was modified, and the free enzyme released in the liquid phantom was determined with the aim of establishing the relationship between  $[S_0]$  with  $[P]$ , and  $\alpha$  values were calculated to evaluate the role of CBDs in the adsorption of cellulases onto fibers.

#### *Digestion of cellulase B to prepare CBDs*

CBDs were prepared by digestion of cellulase B with papain as described by Lemos et al. (2000). The ratio of cellulase to papain (Solarbio Science & Technology Co., Ltd., Beijing) was 300:1 (w/w), with the digestion performed at 25 °C for 3 h. The digested mixture was ultrafiltrated through a 10-kDa nominal cutoff membrane (Solarbio Science & Technology Co., Ltd.) to separate the CBDs from the digested mixture. The separated CBDs and cellulase without CBDs were tested for their protein content and activity (Table 1).

#### *Enzymatic beating process and determination*

The pulp suspension was warmed up to 45 °C, and the pH level was adjusted to 5.0 using sulfuric acid. Enzymes (2 mg/g oven-dried fibers) were subsequently added and stirred every 10 min in a thermostatic water bath. After the enzymatic treatment, the pulps were filtered in a Buchner funnel, washed with deionized water to neutral pH, and then stored at 4 °C to prevent further enzymatic reactions. The control pulp samples were prepared following the same procedure but without the addition of enzymes.

All pulp samples were refined in a PFI mill (P40110.E000, PTI, Austria) for certain revolutions with a pulp consistency of 10% and a refining intensity of 3.33 N/mm (ISO 5264-2). Measurements of the beating degree, tensile index, tear index, and water retention value (WRV) were carried out in accordance with ISO 5267-1, ISO 1924-2, ISO 1974, and ISO 23714:2007(E) standards, respectively.

#### *Fiber morphology analysis by scanning electron microscopy (SEM) and fiber quality analysis*

SEM images were obtained using a JSM-6380 scanning electron microscope after a combined dehydration treatment of fibers with increasing ethanol concentrations and freeze dehydration treatment to reveal the external fibrillation of fibers. Fiber length was

determined, and fine contents were evaluated using Fiber Tester 912 (Lorentzen & Wettre, Sweden).

### Crystallinity measurement

The cellulose crystallinity of the pulps was determined based on the diffracted intensity of Cu radiation (1.54056 Å, 40 kV, and 40 mA) using an X'ert Pro MPD X-ray diffractometer (Philips, Holland). The samples were scanned at 5°/min, with the  $2\theta$  values ranging from 5° to 80°. Crystallinity was calculated using the following empirical equation (Segal et al. 1959),

$$CI = \frac{I_{Cr} - I_{Am}}{I_{Cr}} * 100\% \quad (4)$$

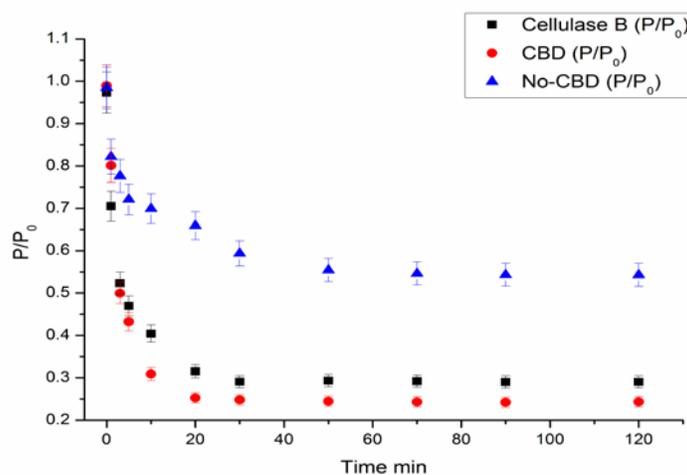
where  $I_{Cr}$  is the average intensity of the crystalline region in the  $2\theta$  range of 22.56° to 22.65° for cellulose I and in that of 21.66° to 21.75° for cellulose II and  $I_{Am}$  represents the average intensity of the amorphous region at the  $2\theta$  range of 18.96° to 19.05° for cellulose I and at that of 15.96° to 16.05° for cellulose II (Kuo, Lee 2009).

## RESULTS AND DISCUSSION

### Isothermal Adsorption of Cellulases onto Fibers: Calculation of the Adsorption Parameters

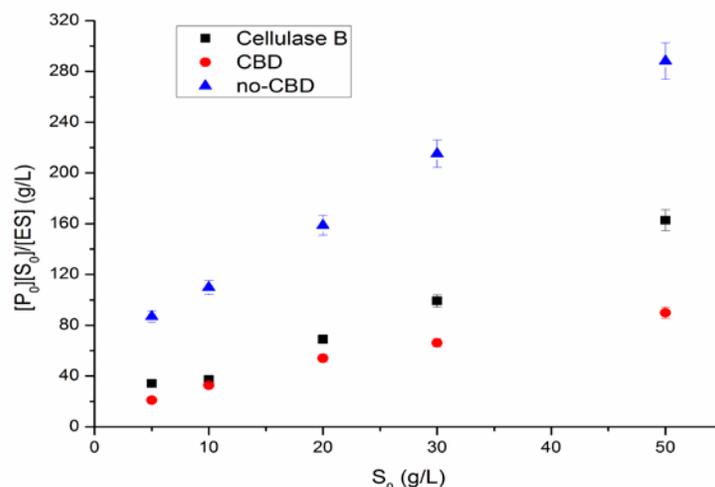
#### Effect of enzyme contact time on the adsorption of cellulases

Different enzyme contact times at a given pulp consistency, enzyme dosage, and temperature were investigated to determine the effect of enzyme contact time on the adsorption of cellulases and to determine the absorption equilibrium time. The adsorption kinetic processes are shown in Fig. 1.



**Fig. 1.** Effect of enzyme contact time on the adsorption of cellulase B, CBDs, and cellulase without CBDs ( $[P_0]=30$  mg/L; pulp consistency, 3%; temperature, 45 °C; pH 5.0)

In Fig. 1,  $[P_0]$  is the total concentration of protein added at the beginning of the adsorption experiments and  $[P]$  is the detected free enzyme concentration in the liquid medium during the adsorption experiments, with  $[P]/[P_0]$  thus indicating the relative adsorption proportion of cellulases onto the fibers. Generally, low  $[P]/[P_0]$  values represent less cellulase being released in the liquid medium, more cellulase being adsorbed onto the fibers, and easier adsorption (Shen 2000). As illustrated in Fig. 1, the adsorption curve of cellulase B (full cellulase; contains CBDs) significantly changed in 20 min and became mild after 30 min. The rate of adsorption approached zero in approximately 30 min, indicating the approach of cellulase absorption equilibrium. However, as the CBDs were removed from the cellulase, the adsorption curve (“No-CBD” in the figure) reached equilibrium after a longer time (ca. 40 min), and the amount of cellulase adsorbed was considerably less than that of the full cellulase. The adsorption curve of CBDs reached equilibrium (20 min) much faster than did the full cellulase and cellulase without CBDs, and the amount of CBDs adsorbed was the highest among the three. These findings suggest that the presence of CBDs in cellulase is crucial to the adsorption of cellulases onto fibers, in agreement with the results reported by Markus (1997), which showed that the adsorption of cellulases was reduced when the CBDs were removed from the protein. Subsequent adsorption experiments were conducted at the fixed contact time of 40 min based on these data.



**Fig. 2.** Effect of fiber substrate consistency on the adsorption of cellulases (where the coordinate axis was set according to Eq. (5))

*Effect of fiber substrate concentration on the adsorption of cellulases: Calculation of the  $\alpha$  value*

The maximum amount of protein that can be adsorbed during an enzymatic reaction is a controlling factor for fiber modification and directly depends on the accessibility of sites on the fiber substrate. Because CBDs are reportedly responsible for the binding of enzymes onto cellulose, the isothermal adsorption of cellulases onto fibers at varying substrate concentrations was examined under specified conditions to further understand the behavior and role of CBDs in cellulases and the adsorption properties of

cellulases. Equation (5), from which  $\alpha$  values can be calculated, could be derived from Eqs. (2) and (3) and expressed as the reciprocal form. In the present study, fiber substrate consistency  $[S_0]$  and enzyme dosage  $[P_0]$  as a function of  $[P]$  were calculated as follows:

$$\frac{[P_0][S_0]}{[ES]} = \frac{K1}{\alpha} + \frac{[S_0]}{\alpha} \quad (5)$$

Based on the linear equations listed in Table 2, all  $R^2$  values reached or were close to 0.99, indicating that the equations obtained had excellent linearity, thereby confirming that  $\alpha$  values can be directly calculated from the slope of the equations. The  $\alpha$  value is the fraction of effective adsorbable enzyme protein accounting for the total water-soluble protein in the enzyme preparation.

**Table 2.** Linear Equations of Cellulase Adsorption

Enzyme	Linear equation	$R^2$	A
Cellulase B	$y = 2.96 * x + 12.45$	0.9928	0.3381
CBDs	$y = 1.50 * x + 18.21$	0.9923	0.6569
No-CBD	$y = 4.55 * x + 67.18$	0.9745	0.2199

Note: Cellulase adsorption was applied as a function of fiber substrate consistency, as expressed in Eq. (5).

As shown in the table, the  $\alpha$  value of cellulase B was higher than that of cellulase without CBDs but lower than that of the CBDs. Research has shown that the removal of the substrate-binding domains of enzymes decreases their adsorption ability and activity on insoluble substrates (Blaak and Schrempf 1995; Mansfield et al. 1999). The low  $\alpha$  value obtained for cellulase without CBDs in the present study indicates that its ability to be adsorbed onto fibers was undermined with the removal of CBDs, whereas the high  $\alpha$  value calculated for CBDs confirms that the presence of CBDs in cellulase (cellulase B) is essential in the adsorption of cellulases onto fibers.

The endoglucanase activity of cellulase without CBDs was close to that of the full cellulase, but its filter paper and cellobiohydrolase activities were significantly reduced, indicating that the cellulase activity on the insoluble substrate (filter paper or fibers) was impaired with the removal of the CBDs from cellulase B but had little effect on the soluble substrate (carboxymethylcellulose solution). Similar evidence from the characterization of cellulase preparations as listed in Table 1 support these findings. Other studies have suggested that CBDs have a high affinity to cellulose, involving the adsorption of enzyme to the cellulosic substrates and having no catalytic activity (Tomme et al. 1998; Xiao et al. 2001). These data clearly reveal that such a modular domain structure offers some significant advances in the adsorption and degradation of insoluble substrates.

### Effect of CBDs on the enzymatic refining energy consumption of NBKP pulp

Enzymatic beating treatments of NBKP pulp were conducted with three cellulase preparations under optimal processing conditions to better reveal the role of CBDs in the modification of fibers. The effects of the three cellulase preparations on enzymatic

refining energy consumption were compared when the pretreated pulps were refined to the same PFI revolutions and when they were refined to the same or a similar beating degree (Table 3). The energy consumption of the refining was read directly from the PFI mill display panel.

**Table 3.** Energy Conservation at the Optimal Enzymatic Treatment Conditions

	PFI Revolutions:40000		Beating degree: 75±1°SR	
	Energy consumption (kWh/t, based on O.D. fibers)	Beating degree (°SR)	Energy consumption (kWh/t, based on O.D. fibers)	PFI Revolutions
Control	15200	67	15933	50500
Cellulase B	12400	77	11933	38500
CBD	14500	72	14833	41000
No-CBD	13600	70	14666	43500

Note: The enzymatic refining conditions were as follows: enzyme dosage, 2 mg protein/g o.d. fiber; pulp consistency, 3%; temperature, 45 °C; enzyme contact time, 35 min; PFI mill refining intensity, 3.33 N/mm.

All three enzymatic pretreatments led to significant refining energy reductions. When the pretreated pulps were refined under the same PFI revolutions (40,000), the samples demonstrated an improvement in their beating degree, ranging from 3° to 10° SR (Schopper–Riegler beating degree). Cellulase B revealed the greatest improvement, whereas cellulase without CBDs demonstrated the least. Although the CBDs demonstrated less or almost no activity on the cellulose, they had favorable effects on the improvement of the beating degree. Moreover, compared with the control sample, the pulps pretreated with cellulase B, CBDs, and cellulase without CBDs and refined to the same or a similar beating degree (75° SR) saved 25.1%, 6.7%, and 8.0% of the refining energy, respectively. In contrast, compared with the control sample, approximately 23.8%, 18.81%, and 13.9% of the PFI revolutions were saved for cellulase B, CBDs, and cellulase without CBDs, respectively. These discrepancies in energy consumption and PFI revolution savings can be explained by the different refining resistance levels or responses of the pulps treated with different enzymes. Although more PFI revolutions can be saved, the higher the refining resistance was, the less the refining energy savings became.

In addition, as shown in Table 3, although all pulp samples were refined under the same PFI revolutions, the energy consumptions of the enzyme samples were lower than the energy consumption of the control sample. Such difference in energy consumption at the same refining revolutions may be attributed to differences in fiber morphology. Enzymatic modification of fibers may be enhanced by the synergic action of cellulase with CBDs in cellulase B, which contributes to the flexibility of fibers, consequently lowering resistance to PFI mill refining. Studies have suggested that the presence of CBDs is beneficial to the adsorption of cellulases onto fibers, thus enhancing the modification of fibers (Pinto et al. 2006; Reinikainen et al. 1997). In addition, the CBDs themselves can be adsorbed onto the cellulose fibers and are able to disrupt the

crystallinity of the cellulose as well as increase the beatability of the pulps (Chunhui et al. 1997). Contrarily, as the CBDs were removed from cellulase, the adsorption ability of the cellulases onto fibers was undermined, rendering less effective enzymatic beating inevitable. Suurnäkki et al. (2000) found better beatability for bleached chemical pulps treated with intact endoglucanases and suggested that the presence of CBDs in endoglucanases may result in beneficial effects on pulp properties, which are instrumental in the development of the beating degree of the pulps. Taken together, these data indicate that the presence of CBDs is beneficial for the cellulase action on fibers and that CBDs in cellulases may help loosen individual cellulose chains as well as enhance the beatability of fibers.

### Effects of Pretreatments with Cellulase and CBDs on the Physical Properties of Handsheets

Tensile index and tear index are important strength properties of paper, and the fiber morphology (fiber length, fine content, and WRV) plays a vital role in the development of the physical properties. Table 4 lists the main strength properties of handsheet and details the fiber morphology, density, and scattering coefficients of the pulp samples.

**Table 4.** Physical Properties of Handsheet and Fiber Morphology

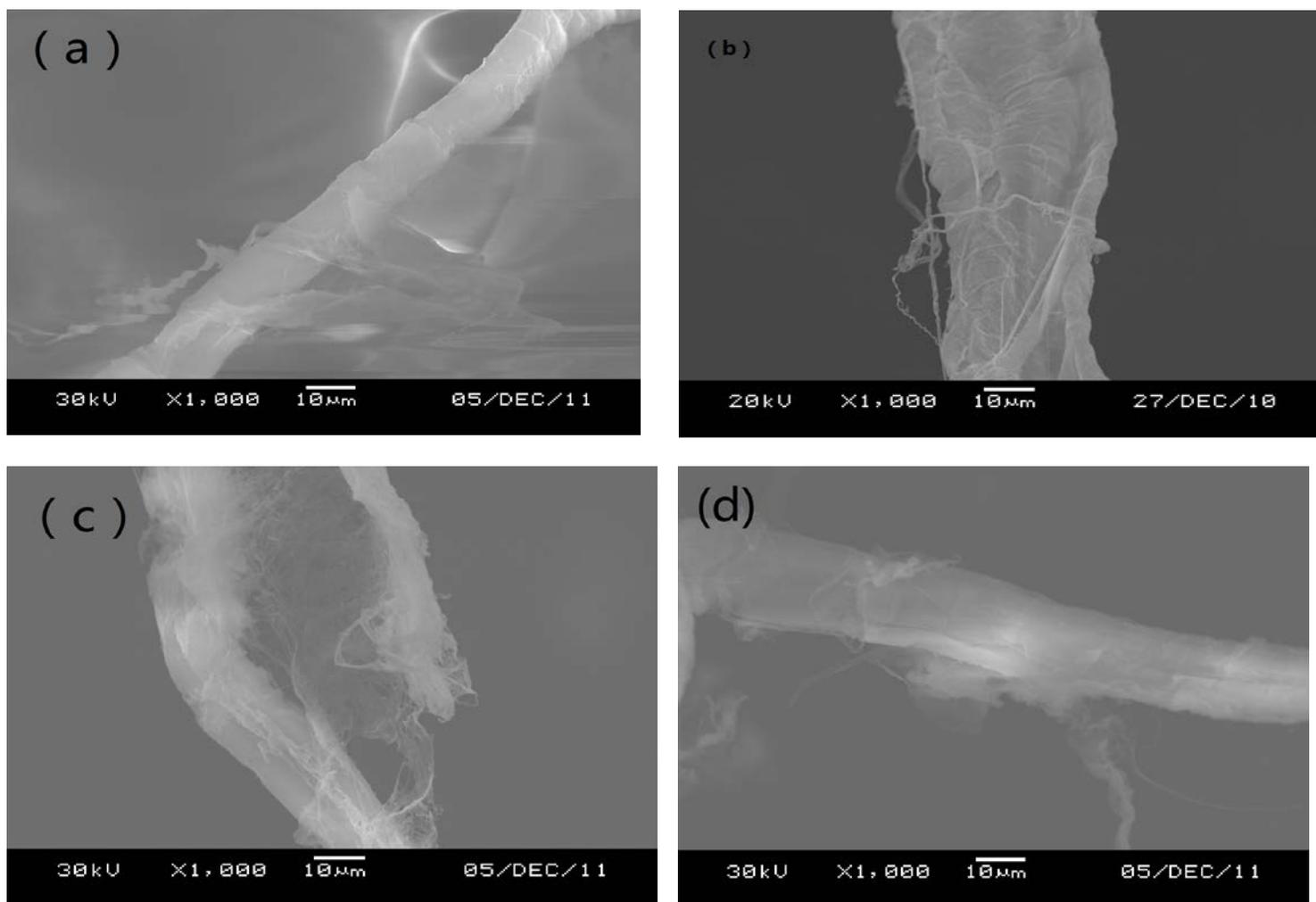
	Tear index (mN · m <sup>2</sup> /g)	Tensile index (N · m/g)	Density (g/m <sup>3</sup> )	Scattering coefficient (m <sup>2</sup> /kg)	WRV (%)	Fiber length (mm)	Fines (%)
Cellulase B	11.34	3.02	0.636	37.9	205	1.10	7.5
CBD	15.64	4.35	0.650	33.0	199	2.09	4.7
No-CBD	13.36	3.32	0.632	36.8	191	1.05	8.5
Control	18.31	3.78	0.611	34.2	182	2.12	4.6

Note: All pulps were refined to the same or a similar beating degree (75° SR).

As shown in Table 4, the tear index of the handsheets decreased after the pulps were treated with cellulase B, CBDs, or cellulase without CBDs; pretreatment with cellulase B damaged the tear index most severely. A similar trend for tensile index was observed for pretreatment with cellulase B and that with cellulase lacking CBDs, whereas pretreatment with CBDs significantly increased the tensile index. These findings were supported by the scattering coefficient and density values of the handsheets, with higher density and lower scattering coefficients suggesting more effective bonding of the fibers in the handsheet. In addition, the unchanged fiber morphology of the fibers pretreated with CBDs, especially fiber length, plays a critical role in the development of the tensile index of the handsheet. López-Lorenzo et al. (2003) reported that the relative bonding area, flexibility, and fibrillation of fibers can be modified by CBDs and by cellulases with CBDs to improve the properties of both fiber and paper. The handsheet tensile and tear indices of the pulps pretreated with CBDs that were obtained in the present study confirm that CBDs can modify the fiber surface by increasing the flexibility and swelling ability (WRV) of the fiber or by splitting the cellulose chain and releasing more active surfaces for bonding, but they also reveal that CBDs may lower the strength of a single fiber and result in some reduction in the tear index.

### Analysis of Fiber Surface Morphology and Degree of Cellulose Crystallinity

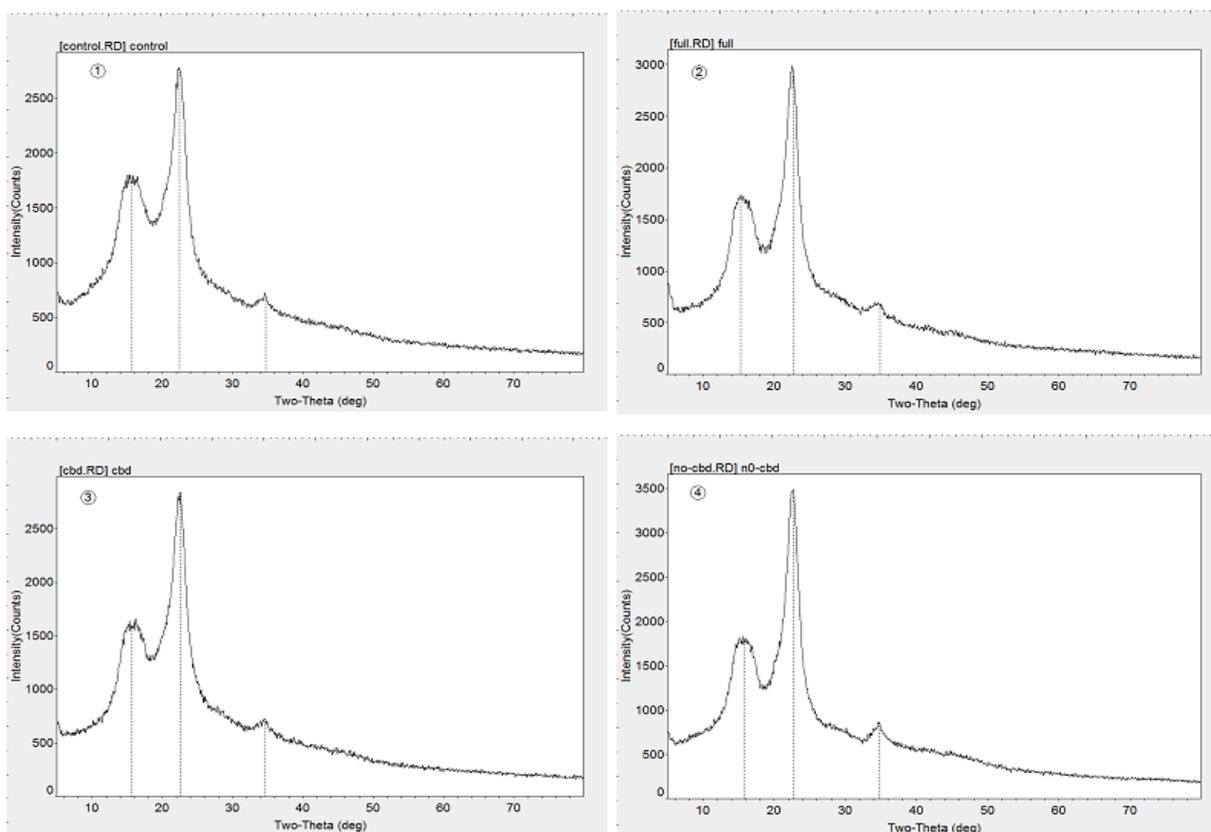
A combined dehydration treatment of fibers with increasing concentrations of ethanol and freezing dehydration was carried out to make SEM observations of the fibers easier. The degree of cellulose crystallinity was studied using X-ray diffraction (XRD) analysis.



**Fig. 3.** SEM images of fibers from (a) the control pulp and (b–d) the pulps pretreated with (b) cellulase B, (c) CBDs, and (d) cellulase without CBDs. All samples were refined in a PFI mill.

As shown in Fig. 3(a), examination of the control fiber by SEM revealed a peeled-off surface of the fiber and no indication of fibrillation. However, when the pulps were pretreated with cellulase B and subsequently subjected to PFI refining, significant changes in fiber morphology emerged (Fig. 3(b)): more fibrils along the fiber and delamination of cell walls were observed. Pretreatment with CBDs also revealed that a peeled-off surface was attached to the fiber. The peeling layers of the fiber increased the surface area, which contributes to interfiber bonding. Pretreatment with cellulase lacking

CBDs caused less peeling of microfibers and the fiber remained somewhat more intact. These observations suggest that cellulase activity enhanced mechanical refining, with more external fibrillation, and that CBD activity softened the bonding of fiber layers as well as the peeling off induced by mechanical refining. The fiber became flat after pretreatment with cellulase B and PFI refining, and the CBDs seemingly helped soften and split the surface layers; however, the full cellulase might have adsorbed and digested these amorphous regions and accessible sites created by CBD activity, thus accounting for the less attached peeling layers with larger specific surface areas, as shown in Fig. 3(b), compared with Fig. 3(c). In contrast, pretreatment with cellulase lacking CBDs and refining yielded less fibrils (Fig. 3(d)) than did pretreatments with cellulase B and CBDs even under the same PFI refining conditions. This finding is attributed to the fact that PFI refining favors internal rather than external fibrillation. Din et al. (1991) reported that isolated CBDs of endoglucanase A (CenA) from *Cellulomonas fimi* disrupted the structure of cellulose fibers and released small particles (Kerekes 2005; Din et al. 1991). Thus, the fiber morphology determined in the present study strongly indicates that pretreatment with cellulase containing CBDs is more effective for enzymatic beating.



**Fig. 4.** XRD patterns of cellulose with and that without enzymatic pretreatment. 1, control sample; 2, sample pretreated with cellulase B; sample pretreated with CBDs; 4, sample pretreated with cellulase lacking CBDs  
All samples were refined under the same PFI revolutions (40,000).

The crystal structures of cellulose have been reported to significantly affect its hydrolysis kinetics (Kuo and Lee 2009). The cellulose crystallinity of the samples used in the present study was determined using an X-ray diffractometer, and the crystallinity index was calculated according to Eq. (4).

**Table 5.** Crystallinity of Cellulose after Enzymatic Beating

	$I_{cr}$	$I_{AM}$	Crystallinity index (%)
Control	2869	1132	60.54
Cellulase B	2918	1310	55.11
CBDs	2709	1268	53.19
No-CBDs	3226	1319	59.11

The XRD patterns of the control and pretreated cellulose samples showed distinct peaks with diffraction angles ( $2\theta$ ) at approximately  $15.5^\circ$ ,  $22.5^\circ$ , and  $34.5^\circ$  (Fig. 4), indicating that their crystal structures consisted of celluloses I and II (Kuo and Lee 2009). After pretreatments with cellulase B and cellulase lacking CBDs, the peak intensities at approximately  $15.5^\circ$  and  $22.5^\circ$  slightly increased due to the limited reduction in crystalline cellulose, with the presence of CBDs in cellulase B resulting in even greater reduction; however, after pretreatment with CBDs, the peak intensity at  $22.5^\circ$  decreased and that at  $15.5^\circ$  increased. Thus, as clearly shown in Table 5, pretreatment of pulp with cellulase B and subsequent PFI refining slightly decreased the crystallinity index (from 60.54% to 55.11%), whereas that with CBDs significantly reduced it (from 60.54% to 53.19%). Pretreatment with cellulase B lacking CBDs had little effect (from 60.54% to 59.11%) on cellulose crystallinity.

Research has shown that crystalline cellulose is much more resistant to hydrolyzation and to refining than amorphous cellulose (Hong et al. 2007; Rahkamo et al. 1998). This finding indicates that the enhanced development of the beating degree of the pulp after pretreatment with CBDs or cellulase B in the present study was caused by the presence of more amorphous cellulose in the restructured cellulose after pretreatment. CBDs clearly play an important role in the adsorption and cracking of the crystalline region in cellulose, and the removal of CBDs from cellulase undermines the ability of the cellulase to adsorb and act on the cellulose fibers. These results are in agreement with those reported by Pinto et al. (2006), Reinikainen et al. (1997), and Chunhui et al. (1997): CBD activity helps the adsorption of cellulases onto fibers and enhances the disruption of crystalline regions for rapid enzymatic reaction, leading to a low cellulose crystallinity index.

## CONCLUSIONS

1. Experiments on the isothermal adsorption kinetics of cellulases onto fibers confirmed that the presence of CBDs in cellulases enhances their adsorption capacity, as reflected by the  $\alpha$  values obtained (0.3381 for cellulase B, 0.2199 for cellulase B without CBDs, and 0.6569 for CBDs).

2. Fiber can be modified by CBD adsorption to improve some of its properties, especially the beatability of the pulp and the tensile index of the handsheets. The increased beating degree could lead to energy savings in the refining process. Approximately 13% to 24% of refining energy was saved when pulps were pretreated with cellulase B, CBDs, or cellulase without CBDs under optimal conditions.
3. Analysis of the physical properties of the handsheets revealed that pretreatment with cellulase is somewhat detrimental to the tear and tensile indices but that pretreatment with CBDs is instrumental in developing the bonding strength of fibers.
4. SEM micrographs of fiber morphology revealed that enzymatic refining with cellulase plus CBDs (cellulase B) caused greater external fibrillation of fibers and fiber lumen collapse.
5. Analysis of the crystallinity indices of celluloses that had undergone different pretreatments confirmed that the presence of CBDs in cellulase B improved enzyme adsorption onto the fibers, thereby splitting the crystalline cellulose and decreasing the crystallinity index.

## ACKNOWLEDGMENTS

This study was supported by the International Scientific and Technological Joint Project of China and Canada (Grant No. 2008DFA91290), the Scientific and Technological Support Program of the Ministry of Science and Technology of China (Grant No. 2011BAC11B04), the Tianjin Technological Innovation and Development System, and the Planned Project of the Condition Development Platform (No. 10SYSYJC28000).

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Article submitted: September 20, 2011; Peer review completed: October 31, 2011;  
Revised version received: December 8, 2012; Accepted: January 9, 2012; Published:  
January 11, 2012.