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Effects of commercial cellobiohydrolase treatment on fiber strength and morphology of bleached hardwood pulp

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Abstract

Development of fiber strength properties and morphological changes on the surface of bleached hardwood kraft pulp after treatment with commercial cellobiohydrolase (CBH) was evaluated. Tensile and tear indices showed no significant effect of the treatment. The treatment resulted in reduction of wet zero-span strength, while the dry zero-span values increased. The decrease in wet zero-span values was most likely caused by preferential action of CBH on structurally irregular zones in the fiber wall coupled with greater sensitivity of wet zero-span testing towards the localized fiber defects. The fracture zone of the wet zero-span tested samples was imaged by scanning electron microscopy (SEM). Visual observation revealed differences in fiber fracture between treated and control samples. The straighter and cleaner fractures of the treated sample could be attributed to the localized hydrolytic action of CBH. Visual analysis of the treated fiber surface morphology by SEM showed an increase of more visible fiber wall dislocations, particularly on fibers of smaller diameter. The increased presence of the fiber dislocation correlates with the decrease in the wet zero-span values of treated samples.

Keywords: cellobiohydrolase; cellulase; fiber fracture; fiber morphology; hardwood; scanning electron microscopy (SEM); zero-span strength.

Introduction

The action of enzymes on cellulosic substrates including wood pulp fibers has been studied extensively. The status of enzyme applications in pulp and paper industry was reviewed by Buchert et al. (1998), Bajpai (1999), and Wong and Mansfield (1999). Viikari et al. (2000) specifically described the application of cellulase systems. The potential benefits of cellulase treatment for mechanical, chemical, and recycled fibers were identified in reduction of refining energy demands for mechanical pulps, modifications of chemical fiber, enhanced de-inking of recycled fibers, and for process water treatment.

The term cellulase refers to a group of several cellulose specific enzymes that differ in their mode of action. Exoglucanases (cellobiohydrolases, CBHs; EC 3.2.1.91) act on the chain termini, endoglucanases (EGs, EC 3.2.1.4) attack cellulose randomly within the chain, and β-glucosidase (EC 3.2.1.21) hydrolyse released cellobiose. The individual cellulases work synergistically, and a cellulase system containing all three types of enzymes can efficiently degrade cellulose (Henrissat 1994).

The cellulase mixture can also have a detrimental effect on the fiber strength properties. Thus, it is essential to control the extent of the hydrolytic action. Aiming at this, the treatment conditions (dosage, treatment time) have to be adjusted and/or the synergism has to be eliminated by separation and application of individual cellulase components. This concept was initially evaluated by Pere et al. (1995). It was found that CBH had a lesser impact on pulp viscosity and released carbohydrates than EG. Strength properties of CBH treated samples were similar to those of control samples. Oksanen et al. (1997) studied the effect of individual cellulase components on bleached softwood kraft pulp (KP). The treatment with CBH did not result in a marked effect on the development of pulp properties during refining. Although the EG treatment improved beating response of the pulp, sheet density, and smoothness, these benefits were compromised by detrimental effect on pulp strength. Other efforts are summarized by Buchert et al. (1998). In general, treatments of pulp fibers with individual cellulases resulted in different effects on fiber properties. CBH treatment was shown to have only a moderate or no marked effect on technical properties and viscosity. In contrast, EG treatment improved some properties; however, a noticeable decrease in viscosity and in pulp strength often accompanied the improvements.

A variety of analytical tools, standard tests, and microscopic techniques have been implemented for studying the cellulase action on cellulosic substrates. Electron microscopy (EM) has been applied in visualization of CBH hydrolysis of microcrystalline cellulose (Chanzy et al. 1983), bacterial cellulose (Samejima et al. 1998), and cotton fibers (Hoshino et al. 1993, 1997). The fiber of wood pulp is a significantly more heterogeneous substrate compared to these model cellulose systems. Duchesne and Daniel (1999) reviewed the microscopic techniques for studying wood fiber morphology. Scanning electron microscopy (SEM) was used as a complementary method to study the effect of enzymatic treatment on softwood KP (Mansfield et al. 1997) and on

surface morphology of pine KP (Dickson and Wong 1998).

Understanding the effects of enzymes on the fiber ultrastructure is a necessary requirement for moving towards a successful implementation of biotechnological steps into pulp production. A better knowledge of CBH action on cellulosic fibers is needed both for fiber modification and total hydrolysis of lignocellulosic materials. The majority of the studies in this regard were conducted on fibers of softwood KP. Hardwood fibers differ in carbohydrate composition, shape and size of fibers, and other properties from those of softwood KP. Although some information on cellulase treatment of hardwood pulp is also available (Kamaya 1996; Rahkamo et al. 1996), the overall extent of the research is limited. Accordingly, the objective of this study was to evaluate the effect of treatments of bleached hardwood KP (obtained from birch) with commercial monocomponent CBH concerning fiber strength and fiber morphology. High resolution SEM analysis of the fiber surface and zero-span testing were the methods of choice. The area of the fracture zone resulting of the wet zero-span test should be imaged by SEM and the images should be interpreted in view of possible differences in the fiber fracture mechanism caused by the CBH action.

Materials and methods

Never dried ECF bleached birch KP from a Finnish pulp mill was treated with a commercial CBH (Ecopulp®Energy, AB Enzymes, Finland) designed for TMP reject treatment. The enzyme is a family Cel7A CBH from a thermophilic ascomycetous fungus *Thermoascus aurantiacus* (Viikari et al. 2007). The main enzyme activity in the product is CBH, but it also shows some xylanase activity. Cellulase activity was measured with a commercial fluorescent substrate 4-methylumbelliferyl-β-D-lactoside (Sigma M-2405) at 0.5 mM concentration level, at 70°C in 50 mM acetate buffer (pH 5.0). The fluorescence was measured with a Chameleon multiplate reader (Hidex Oy, Turku, Finland), excitation at 355 nm, and emission at 460 nm. The CBH activity (12 nkat mg^{-1}) was calculated from the difference of cellulase activity analyzed in the presence and absence of 5 mM of cellobiose (Fluka 22150) added to the reaction mixture to inhibit the CBH activity. The total cellulase activity was 14 nkat mg^{-1}. Protein concentration was determined with Bradford reagent (Bio-Rad protein assay) according to the manufacturer's instructions. The absorbance at 595 nm was measured by a Chameleon multiplate reader (Hidex Oy, Turku, Finland). Xylanase activity (37 nkat mg^{-1}) was analyzed by quantifying reducing sugars released from 1% birch xylan (Fluka 95588) substrate with 3,5-dinitrosalicylic acid (Sigma D-0550) (Bailey et al. 1992), at 70°C in 0.1 M citrate phosphate buffer (pH 7.0). Absorbance was read with Shimadzu UV-240 (Shimadzu, Kyoto, Japan) spectrophotometer at 540 nm.

The CBH dosages were 100, 300, and 900 g of protein per ton of pulp (oven-dry basis). The treatments were carried out at 70°C, pH 6.0, 4% consistency, and 60 min retention time. The pH and conductivity (70 mS m^{-1}) of the pulp slurry were adjusted with 0.1 M NaOH (Oy FF chemicals, CAS 1310-73-2) and 0.1 M $MgSO_4$ (p.a. Merck), respectively. Released carbohydrates in the filtrates were analyzed after pH adjustment and acid hydrolysis with 72% sulfuric acid performed according to Hausalo (1995). The monosaccharides were separated in an anion exchange column (Dionex CarboPac PA10, width 4 mm, length 250 mm) and quantified by pulsed amperometric detection (HPAEC-PAD) with Dionex DX-500 (Dionex Corporation, Sunnyvale, CA, USA) liquid chromatograph.

The pulp was refined with Voith Sulzer LR1 (Ravensburg, Germany) industrial type laboratory refiner equipped with disk fillings (2/3-1.46-40D). The specific edge load was 0.5 J m^{-1} and the pulp was refined to three specific energy consumption levels (SEC 0, 25, 50 kWh ton^{-1}). Handsheets were prepared according to EN ISO 5269-1 and tested according to EN ISO 5720 and EN ISO 1924-2 (tensile index), EN ISO 5270, ISO 1974 (tear index), and ISO 15361 (zero-span) standards.

The Hitachi S-3400N SEM (Hitachinaka, Japan) was used for SEM analysis. The samples were gold-sputtered and then the images were taken in secondary electron imaging mode with acceleration voltage 5 kV. Wet zero-span samples were freeze-dried before coating.

Results and discussion

Dissolved carbohydrates

The efficiency of enzymatic hydrolysis of cellulosic matter is most commonly expressed by the quantity of dissolved carbohydrates that are released from the treated substrate (Table 1).

The activity analysis and the evaluation of a preliminary pulp treatment at 50°C revealed the presence of a partial xylanase activity in the commercial CBH. The main treatments were thus carried out at 70°C to ensure optimal action of the thermotolerant CBH and to eliminate any undesirable side activities, such as those of xylanase. The impact of xylan removal on fiber properties should be impeded. However, some xylanase activity was still retained even at 70°C.

It is evident that only a small amount of glucose was released during the treatment. A low hydrolytic efficiency of CBH alone has been reported previously, but the released glucose levels in the present work are up to tenfold lower than those reported for softwood bleached pulps (Pere et al. 1995; Oksanen et al. 1997). In addition to moderate enzyme dosages and short treatment time, several other factors may be responsible for the lower glucose release. First of all, birch fibers contain large amounts of hemicelluloses on their surface, and thus the low amount of released glucose is cogent (Dahlman et al. 2003). The hemicelluloses on the surface can also affect the accessibility of the bulk cellulose and the available surface area, and thus reducing the overall rate and extent of hydrolysis (Mansfield et al. 1999).

Table 1 Carbohydrates released by CBH treatment.

	Dissolved carbohydrates (% of dry pulp weight)		
Dosage (g ton^{-1})	Glu	Xyl	Total
Reference	0.02	0.11	0.13
100	0.03	0.12	0.15
300	0.05	0.13	0.18

Evaluation of pulp strength properties

Tensile index is an indicator of the overall sheet strength and encompasses contributions from individual fiber strength and interfiber bonding (Page 1969). The comparison of measured tensile indices of all samples is presented in Figure 1.

The tensile indices at a given density were similar for both treated and control samples at both levels of refining. The values correlated well, in particular at lower density values, which correspond to levels of no refining and low refining for both CBH treated (at both dosages) and control samples. This indicates that the overall sheet strength of the treated pulp was not affected by the CBH treatment.

Tear index at a given tensile index value is an indicator of pulp or paper web strength and it is commonly expressed in the form of a tear-tensile indices correlation. The correlation is illustrated in Figure 2.

The samples treated at low CBH dosage did not exhibit a marked difference in tensile-tear relation and correlated well with that of the control sample. The treatment at higher CBH dosage resulted in a slight decrease of the tear index values at a given tensile index. Although the decrease was relatively small, the difference was consistent for both levels of refining. These observations agree with the development of tensile and tear indices previously reported for softwood pulps (Pere et al. 1995; Kibblewhite and Clark 1996; Oksanen et al. 1997). In conclusion, the actual impact of the CBH treatment on the sheet strength properties of the pulp studied is very small.

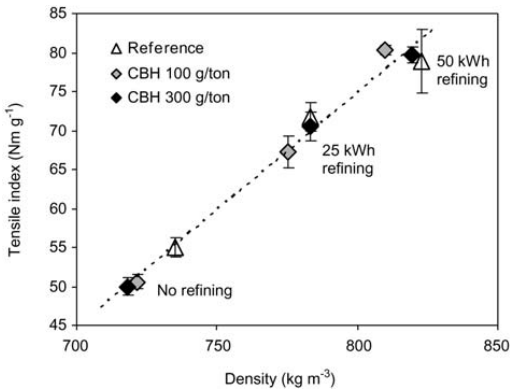

Figure 1 Tensile index vs. apparent density relationship for control and CBH treated pulp.

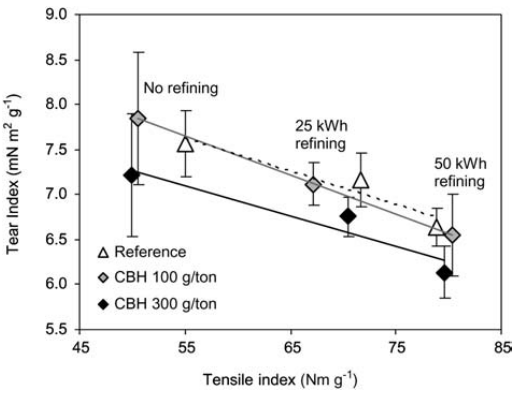

Figure 2 Tensile and tear indices correlation.

Fiber strength properties

The zero-span test is a direct measure of actual strength of individual fibers and can be measured for wet and dry samples (Figure 3a and b).

The wet zero-span testing (Figure 3a) shows a decrease in the measured values of the CBH treated samples at all refining levels. The value decreased with increasing enzyme dosage. Accordingly, the wet strength of the individual fibers is affected by the treatment even at a low level of CBH dosage and in the case of a minuscule amount of released glucose. A decrease in wet zero-span values as a result of extensive cellulase treatment of softwood pulp was reported previously (Mohlin and Pettersson 2002). It has been demonstrated that the zero-span tensile index of rewetted samples is more sensitive to the localized cellulose degradations. The stress transfer between fibers that occurs in the dry state is eliminated, and thus the impact of fiber defects is greater in the wet state (Mohlin et al. 2003). The structurally irregular zones in the fiber wall are more susceptible towards enzymatic attack, which in turn affect the strength of the fiber (Gurnagul et al. 1992). Ander (2002) and Nyholm et

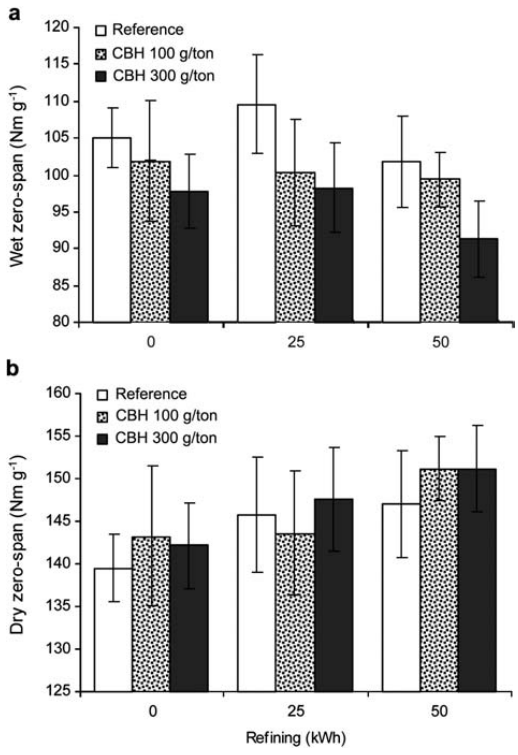

Figure 3 Effects of CBH treatment and refining on wet (a) and dry (b) zero-span tensile indices.

al. (2001) proposed that irregular zones in the fiber wall are the preferential sites of cellulase action and dislocations also occur at these sites.

The dry zero-span measurements (Figure 3b) show an opposite tendency. The CBH treated samples have slightly higher values than the control samples. Though relatively small, the trend is evident and consistent for both refined and non-refined samples. The dosage did not appear to have a pronounced effect on the increase in the measured values. An increment of the dry zero-span value after refining was predicted by Page (1985) and Seth and Chan (1999), and according to Mohlin et al. (2003) the impact of the fiber wall dislocation and localized cellulose degradations diminishes with drying. Nevertheless, the slight increase in the dry zero-span data after the CBH treatment was unexpected.

Zero-span fiber fracture zone evaluation

To better understand the effect of CBH treatment on individual fiber strength, the tested wet zero-span strips were evaluated by SEM. The measurement requires samples to be completely dry. To prevent fiber alteration possibly caused by drying, the wet samples were freeze-dried. The objective was to image the fracture zone of the tested strips at high magnification level (2000×) and visually evaluate possible differences in fiber fracture mechanism. This approach has previously been reported as an internal publication (P. Ander personal communication, February 2009). The SEM images of the fracture zone of CBH treated (300 g prot. ton^{-1}) and control samples are presented in Figure 4.

The comparison revealed differences in areas showing fiber fracture. On several occasions, the fractures of the CBH treated fibers appeared to be straighter in transverse direction. The arrows in Figure 4 indicate examples of such fractures. Several representative magnified images of these fractures, indicated by frames in the main SEM micrograph, are shown in the lower part of the Figure. Although these types of fractures could also be found in the control sample, their increased presence in the CBH treated sample was evident.

The fracture type of the control sample was different. The fiber fractures did not appear to be straight, and on several occasions the outermost layer of the fibers was peeled or disrupted. The rupture zones of the individual fibers was much longer in axial direction, as shown in more detailed images in the lower part of Figure 4 (right-hand side).

It could be speculated that the stress applied to break the untreated control fiber is dissipated over the whole fiber, and thus the fiber can absorb a greater amount of energy. Consequently, more energy is required to rupture or break the fibers. On the contrary, the enzyme treated samples are affected by the localized hydrolytic action of the CBH, which preferably targets the dislocation sites on fiber surfaces (Nyholm et al. 2001; Ander 2002). These localized points of cellulose degradation present the weak spots of the fiber. Once the fiber structure is disrupted at one point, the overall integrity and intrinsic strength of the fiber is compromised, leading to lower resistance toward the applied stress (zero-span value) and possibly resulting in a cleaner cut of the fibers.

Fiber surface morphology

The visual assessment of cellulose model substrates clearly demonstrated the hydrolytic effect of cellulases (Chanzy et al. 1983; Hoshino et al. 1993, 1997; Samejima et al. 1998). The corresponding optical changes on surfaces of more complex wood pulp fibers were less evident after treatment with enzymes under industrially

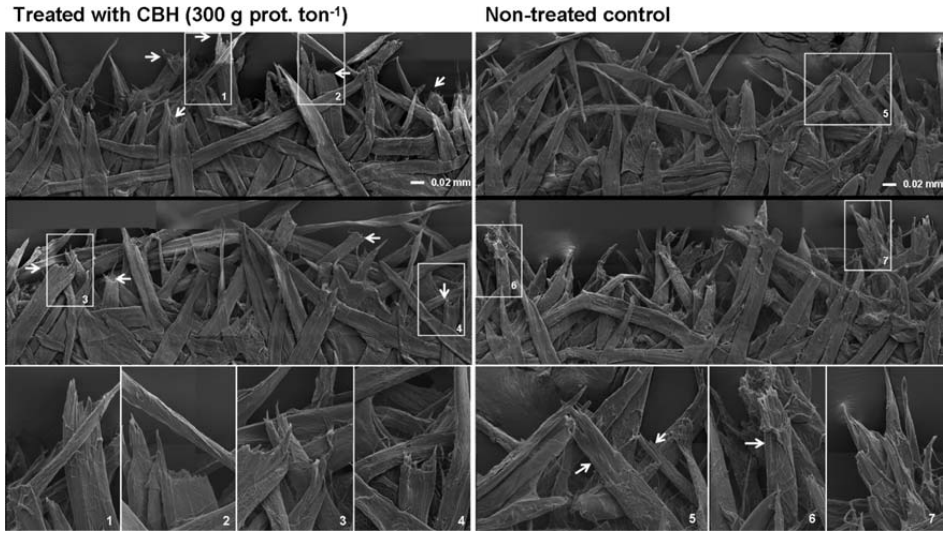

Figure 4 SEM images from the rupture zone of wet zero-span testing. Left: CBH (300 g prot. ton^{-1}) treated sample. Arrows indicate fibers with clean, straight fractures, hypothetically ascribed to the impact of CBH. Right: Non-treated control sample. Arrows indicate examples of fracture type predominant in control sample. In both cases, frames in the image represent magnified areas depicted in the lower part of the Figure.

applicable conditions. SEM as a complementary method to the physical characterization of enzymatically modified softwood kraft fiber did not reveal any distinct alteration to the fiber surface (Mansfield et al. 1997). Similarly, no substantial effect on fiber morphology was observed on softwood KP fibers treated by CBH to modify their papermaking properties (Dickson and Wong 1998).

In this evaluation, the CBH dosage and retention time were moderate in order to comply with the dosages intended for industrial application. To exaggerate the visibility of possible fiber surface alterations, an additional treatment at 900 g prot. ton^{-1} CBH charge was carried out. Representative SEM images of each dosage trial are shown in Figure 5a–c.

The initial observation of SEM images of CBH treated samples (100 and 300 g prot. ton^{-1}) did not reveal any distinctive surface alterations. However, compared to the reference sample, a noticeably greater amount of more apparent fiber wall dislocations was observed. Examples of these dislocations are shown in Figure 5a and b. The dislocations preferably occurred on fibers of smaller diameters. The fibers of greater diameters and vessels did not appear to be affected. A closer examination of the images acquired at higher magnification showed that the outermost layer of the fiber wall was not interrupted and remained intact. This could indicate that the CBH possibly penetrates the outer layer of the fiber at the sites of original dislocations and proceeds with hydrolytic action in the inner layers of the fiber.

The higher enzyme dosage treatment (300 g prot. ton^{-1}) resulted in somewhat increased occurrence of the fiber dislocations compared to the low enzyme dosage sample. The sample treated with the highest CBH dosage (900 g prot. ton^{-1}) showed, in addition to an increase in the number of dislocations, occasional disruptions in the form of cracks on the fiber surface (Figure 5c). It appeared that the outer layer of the cell wall was interrupted in several places, possibly exposing the S2 layer of the fiber wall. One can envisage that these cracks are enlargements of structural irregularities which are originally present in the fibers prior to the enzymatic treatment. Indeed, Gurnagul et al. (1992) have previously suggested that the structurally irregular zones on the fiber surfaces are more susceptible towards enzymatic attack resulting in localized sites of degradation. In addition, the localized action of cellulases preferentially acting on dislocations in softwood KP fibers was clearly visible by polarized light microscopy (Ander et al. 1996; Ander 2002).

The SEM analysis of hardwood pulp fiber handsheets did not allow for a proper quantitative analysis of the dislocation occurrence on the fiber surface. However, their increased visibility and occurrence after the CBH treatment observed visually correlates well with the results of tear index and wet zero-span measurements. In both instances, the decrease in measured values could be related to the increase in number of fiber wall dislocations.

Conclusions

The CBH treatment did not affect the overall handsheet strength properties, but the strength of individual fibers was affected.

The tensile indices of the pulp were at a given density similar for treated and control samples at both levels of refining and non-refined samples. The higher dosage CBH treatment resulted in a slight decrease in the tear index values at a given tensile index.

The weakening of single fiber strength was obvious from the wet zero-span testing. The performance of untreated control samples was better than that of CBH treated samples at all refining levels. This is most likely due to preferential action of CBH on structurally irregular zones in the fiber wall, and wet zero-span testing has great sensitivity towards the localized fiber defects. In contrast, the results of dry zero-span tests are marginally increased after the CBH treatment. The increase was consistent through both refining levels.

The SEM analysis of the wet zero-span tested strips revealed different fracture types of control samples and CBH treated samples. The fractures of the latter appeared to be straighter and cleaner as a result of localized hydrolytic action of the CBH. The preferential attacks on sites of fiber dislocations created weak spots in the fibers. These spots weakened the integrity of the

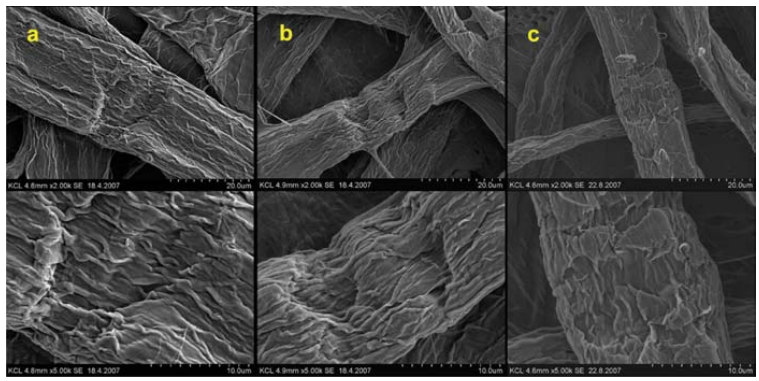

Figure 5 SEM images of CBH treated samples: dosage – 100 (a), 300 (b), and 900 g prot. ton^{-1} (c); magnification: 2000× (top) and 5000× (bottom).

overall fiber strength in the wet state, leading to cleaner and straighter fiber fractures.

Dislocations and disruptions were detected on the fiber surfaces after CBH treatment. SEM analysis of the morphology of treated fiber surfaces revealed more visible fiber wall dislocations, particularly on fibers of smaller diameter. In addition to the dislocations, disruptions in the form of cracks on the fiber surface were occasionally observed on fibers treated with the highest CBH dosage. The increased occurrence of these dislocations correlates with the decrease of wet zero-span strength.

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