Studies on the Cellulose-Binding Domains Adsorption to Cellulose

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Cellulose-binding domains (CBD) are modular peptides, present in many glycanases, which anchor these enzymes to the substrate. In this work, the effect of CBD adsorption on the surface properties of a model cellulose, Whatman CF11, was studied. The methods applied include inverse gas chromatography (IGC), ESCA, X-ray diffraction, and scanning electron microscopy (SEM). The CBD partition affinity (0.85 L/g) was calculated from adsorption isotherms. However, true adsorption equilibrium does not exist, since CBDs are apparently irreversibly adsorbed to the fibers. Both IGC and ESCA showed that fibers with adsorbed CBD have a lower acidic character and also a slightly higher affinity toward aliphatic molecules. This may however be a consequence of an increased surface area, a hypothesis that is supported by microscopic observations. The crystallinity index was not affected by CBD treatment.

Introduction

The cellulose degrading enzymes (cellulases) are constituted with three domains: the catalytic domain, a highly O-glycosylated linker region, and a carbohydrate-binding module (CBM). Several works have shown that the presence of CBM is essential for the enzyme binding to insoluble substrate and, consequently, for its efficient degradation.[1−3] In the soluble substrates, the presence of the CBM does not affect the enzyme activity.[4,5] Deletion of the linker reduces the enzyme activity over insoluble cellulose, even if adsorption still occurs.[6]

According to the amino acid sequence homology, CBMs are classified in more than 30 families,[7] the cellulose binding domains (CBD) being distributed in the first thirteen. All of the identified fungi CBDs belong to Family 1 and have between 36 and 40 amino acid residues. Even if the amino acid sequence in this family is highly conserved, significant differences have been found between several of these CBDs. For instance, Carrard and Linder[8] reported that cellobiohydrolase I and II, from *Trichoderma reesei*, adsorb reversibly and irreversibly to bacterial microcrystalline cellulose (BMCC), respectively. These CBDs apparently adsorb to different sites on the substrate, according to Carrard et al.:[9] a catalytic domain from a *Clostridium thermocellum* endoglucanase (CelD) was fused by these authors to different CBDs. After a certain period of BMCC hydrolysis with one of the hybrids, the reaction is boosted adding another fused protein to the reaction mixture, but further addition of the same hybrid does not improve the reaction rate.

In previous works,[10,11] we reported that the treatment of old paperboard containers with CBDs may increase the pulp drainage rate and the paper resistance index, an effect that was attributed to surface/interfacial modifications. Similarly, Levy and colleagues showed that a CBD from *Clostridium cellulovorans* improves the mechanical properties of Whatman paper sheets, an effect that was even more significant when a double CBD was used.[12] It becomes obvious that it is important to understand the kind of modifications cellulose fibers suffers following CBDs adsorption. Indeed, such effects may have technological significance, since it may be relevant in different fields where cellulases are used, such as paper and textile industries. So far, the effect of glycanases on the treatment of wood and cotton fibers was analyzed always in the perspective of the hydrolytic phenomena. Although relatively low amounts of protein are used in these treatments (0.05−0.5 mg/gr fiber), paper and textile fibers have a relatively low surface area, and thus the adsorbed protein may have a significant effect on the fibers interaction. It is the purpose of this work to study the effect of CBD adsorption on the surface properties of the model cellulose Whatman CF11. Inverse gas chromatography (IGC), ESCA, and adsorption isotherms were used to characterize this system.The IGC theory is well displayed in Schultz and Lavielle[13] and in a more recent review by Belgacem and Gandini.[14]

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Table 1. Characteristics of the IGC Probes[a]

probes	characteristics	γ^D_L (mJ/m^2)	a (Å^2)	$T_{eb.}$ (°C)
hexane	neutral	18.4	51.1	68.7
heptane	neutral	20.3	57.0	98.5
octane	neutral	21.3	63.0	125.6
decane	neutral	23.4	75.0	174.1
chloroform	acidic	25.9	44.0	61.1
THF	basic	22.5	45.0	65.0
diethyl ether	basic	15.0	47.0	34.5
acetone	amphoteric	16.5	42.5	56.0
ethyl acetate	amphoteric	19.6	48.0	77.1

[a] According to Belgacem and Gandini,[14] Belgacem et al.,[16] and Liu and Rials.[17]

Experimental Section

Materials. Whatman CF11 (lote cat. n° 4021050) cellulose fibers were used. CBDs were obtained by the method described in Lemos et al.[15] The probes used for inverse chromatography were *n*-hexane, *n*-heptane, *n*-octane, and *n*-decane, chloroform, tetrahydrofuran (THF), diethyl ether, acetone, and ethyl acetate; all chemicals were of the highest purity available.

ESCA, X-ray Diffraction, and Scanning Electron Microscopy (SEM). Suspensions of Whatman CF11 were prepared by mixing 10 μg of CBD, per milligram of fiber, in 1 mL of sodium acetate buffer (50 mM, pH 5.0). Blanks were prepared similarly, but without CBDs. The incubation was carried out in a thermostatic bath at 50 °C, with agitation, for 12 h (SEM) and 5 days (ESCA and X-ray diffraction). Afterward, the fibers were centrifuged at 10000 g for 10 min, frozen at −80 °C, and lyophilized.

The XPS spectra were obtained in a VG Scientific Escalab 200A, with Pisces data acquisition control software. A nonmonochromatic X-ray source was used with an aluminum anode operating at 15 kV/300 W; for the detailed spectra the analyzer was operated with passage energy of 20 eV. The X-ray diffraction was obtained using a diffractometer (Philips PW-1710) with an automatic divergence slit; a Cu anode with a wavelength of 0.154 nm was used, and the spectra were obtained at 30 mA and 40 kV. For the SEM observation, the fibers were coated with gold particles (Fisons Instruments Polaron SC502 Sputter Coater) and examined in a Leica Cambridge S360 microscope.

CBD Affinity and Binding Reversibility. Suspensions of Whatman CF11 fibers, with a concentration of 10 mg per mL of sodium acetate buffer (50mM, pH 5.0), in the presence of different CBD concentrations ($CBD_{Inicial}$), were incubated for 16 h at 5 °C, with magnetic agitation. Afterward, the fibers were centrifuged at 4000 rpm for 10 min and the CBD concentration in the supernatant ($CBD_{Unbound}$) was measured in a Jasco FP6200 spectrofluorimeter, operated at an emission and excitation wavelengths of 341 and 275 nm, respectively. The apparatus was calibrated using CBD solutions with concentration determined using the BCA protein assay, from Pierce. The bound CBD was calculated using the following equation:

$$CBD_{Bound} = \frac{[CBD_{Initial}] - [CBD_{Unbound}]}{m_{CF11}} \cdot V_R \ (\mu mol_{CBD}/g_{CF11})$$

where V_R (L) corresponds to the volume of buffer used and m_{CF11} (g) to the fibers mass.

Desorption assays were conducted to study the reversibility of the CBD-cellulose interaction. A CBD solution with a concentration of 35.9 μM was allowed to equilibrate with CF11 fibers for 16 h (to reach the equilibrium); the suspension was then diluted by 3-fold and left in contact for different periods of time, from 1 to 32 h. The CBDs concentrations in the supernatant were quantified as described previously.

Chromatography. The measurement of the retention volumes of the several probe molecules (Table 1) was carried out using a Chrompak CP9001 chromatograph, equipped with a flame ionization detector (FID). The several samples of cellulosic fibers to be analyzed were packed in a stainless steel column (Chrompack cat. n° CP99911C), one meter long and with 4 mm of internal diameter.

Table 2. Results of the XPS Analysis

		CF11 with CBD		CF11 without CBD	
	peaks	binding energy (eV)	% area	binding energy (eV)	% area
carbon 1s	C_1	285.0	18.3	285.1	13.0
	C_2	286.7	61.3	286.7	68.9
	C_3	288.4	20.4	288.4	18.1
oxygen 1s	O_1	532.8	20.2	532.9	17.9
	O_2	533.1	79.8	533.1	82.1
nitrogen 1s	N_1	400.2	94.2	400.0	64.5
	N_2	402.3	5.8	402.9	35.5
	O/C	0.636		0.661	
	N/C	0.021		0.009	
	$C_2/(C_1+C_3)$	1.58		2.21	

The Whatman CF11 fibers were washed in 25 mL of acetate buffer (50mM, pH5.0) per gram of fiber for 30 min at 50 °C, with magnetic agitation. These fibers were used as blank. For the CBD treated cellulose the same procedure was applied, but using acetate buffer with a CBD concentration of 10 mg per gram of fibers. Afterward, the fibers were centrifuged at 4000 rpm for 2 min, washed with the same volume of distilled water, and again centrifuged. The fibers were then lyophilized. Replicates were prepared using both the blank and CBD-treated fibers. The exact mass of fibers in the column was weighed (about 6 g in each column).

The flow rate of the carrier gas (helium) was adjusted to 11−12 mL/min, using a soap bubble flowmeter. Both columns were conditioned, first for 2 h at 105 °C, then by applying temperature gradients from 35 to 70 °C, with a step of 3 °C/minute, for 12 h. The retention data were collected for several probes at 35 °C, 42 °C, and 50 °C. A Hamilton Gastight 1750SL syringe was used to inject a 50 μL volume of each probe, mixed with the noninteractive marker (methane). A minimum concentration of the probes was used, to keep the infinite dilution assumption true. At least four analyses were carried out with each probe at each temperature.

Results and Discussion

ESCA. Comparing the CBDs treated fibers with the respective controls, the more obvious difference in the ESCA spectra (Figure 1, Table 2) is the N_1 peak area. The relative proportion of this peak recorded at 400.0 eV increases from 64.5 to 94.2% following the CBDs adsorption; the total area of nitrogen raises by more than 2-fold. The N_1 peak corresponds to nitrogen in a neutral amine ($-NH_2$) and peak N_2 to the protonated ammonium nitrogen ($-NH_3^+$),[18,19] meaning that the adsorbed CBDs raised the concentration of neutral amines on the fibers surface, as expected. The presence of nitrogen on the control fibers is surprising, but not totally unexpected, since the fibers are obtained from wood and, being as large as shown in Figure 4, the extraction of noncellulosic materials is likely not to be complete.

Another spectrum modification is the relative increase of peak C_1 in the presence of the CBD. This peak corresponds to aliphatic C−C and C−H links that should not exist in pure cellulose, therefore revealing the presence of impurities, probably lignin or extractives. The O/C values (0.661 and 0.636, respectively, without and with CBD) are lower than the theoretical value for pure cellulose of 0.83.[19] A similar result was described for other cellulosic

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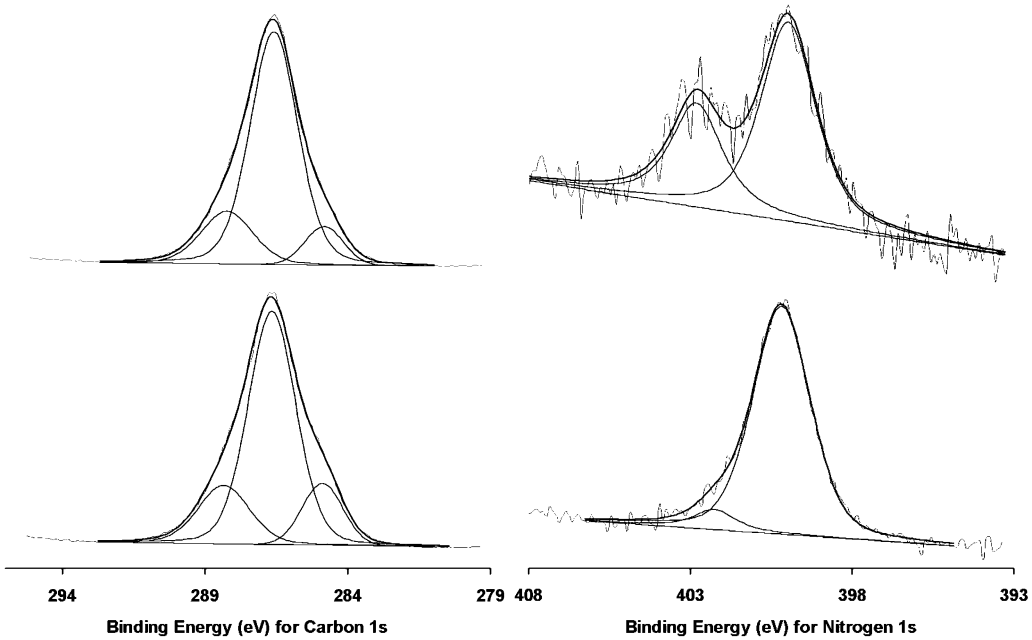

Figure 1. XPS spectra obtained for the CF11 with (bottom) and without (top) CBD.

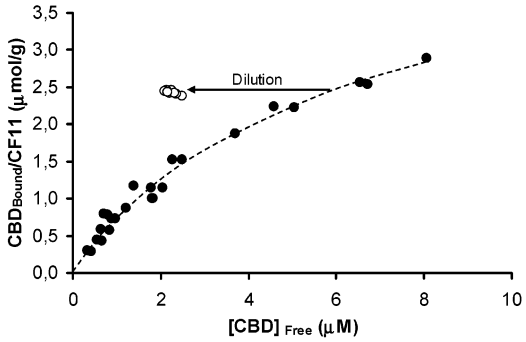

Figure 2. Adsorption isotherm of CBDs on CF11. The open circles correspond to the points obtained during the desorption assays, which were done for 1−32 h.

materials such as C8002, a commercial product of Sigma Co.[16] In that case, values of 0.66 and 0.80 were obtained, respectively, before and after extraction with acetone. Again, these results suggest that commercial celluloses are not totally pure. According to Shen et al.,[20] peak C_2 relates to the fiber acidity, and peak C_1 and C_3 to its basicity. Thus, the ratio of $C_2/(C_1+C_3)$ may provide a measure of the relative acid−base character of the fibers. According to the results, the presence of CBD reduces that value (Table 2) and thus the fibers acidity.

CBD Affinity and Binding Reversibility. The adsorption isotherm at 5 °C, obtained using CBD solutions with initial concentrations between 5.3 and 42.6 μM, is presented in Figure 2. The partition coefficient of 0.85 L/g was calculated from the initial slope of the isotherm. Linder and Teeri[21] obtained a value of 4.9 L/g for the adsorption of cellobiohydrolase I CBD to BMCC, at 5 °C, and Palonen et al.[2] of 2.7 and 1.2 L/g, respectively, for the CBDs of cellobiohydrolase I and II. The much higher surface area of BMCC, as compared to CF11, may explain the differences between these values and the one obtained in this work. As shown in Figure 2, the equilibrium reached after 16 h between the soluble CBDs and the adsorbed ones is not a true equilibrium. Indeed, it appears that most CBDs are irreversibly bound to the fibers, since dilution does not lead to desorption. This is unexpected because the CBDs used in this work where produced from *Trichoderma reesei* cellulases. This cellulolytic system contains four major proteins: cellobiohydrolases I (60−70%) and II (20%) and endoglucanases I and II.[6] Several adsorption studies[8,21] carried out with the CBDs of CBHI and CBHII on BMCC (a crystalline cellulose, as CF11), showed that the first adsorb reversibly, and the second irreversibly. Being a mixture of CBDs, the peptides used in this work were expected to adsorb reversibly, at least partially, which seems not to be the case. The CBD-adsorption assays described by different authors are performed with different kinds of fiber. This may originate the apparently contradictory behavior regarding the binding reversibility. In any case, this irreversibility should not imply that the CBDs are immobile on the cellulose surface. Jervis et al.[22] studied the surface diffusion of a CBD from *Cellulomonas fimi*, that showed irreversible binding to a microcrystalline cellulose, although more than 70% of the bound molecules were mobile on the cellulose surface. This phenomenon should be a characteristic of most if not all CBDs, otherwise the cellulases would not efficiently hydrolyze cellulose.

IGC, Cristallinity, and SEM. The chromatographic peaks obtained with the *n*-alkanes are symmetrical, whereas for the polar probes a tail was observed for all temperatures, both with the treated and untreated cellulose. This phenomenon has been described as resulting from the surface energy heterogeneity of the active sites at the fibers surface.[23] If a simple model with two energy sites surface (high and low) is considered, it is expected that the probe molecules would interact stronger with the high energy sites, producing a wider and flatter peak, and weakly with the low energy ones, generating a sharp and narrower peak. The sum of these two peaks will result in an asymmetrical one,[24] exhibiting a tail, as is observed in the present case.

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Table 3. Specific Retention Volumes ($V_N°$, mL/g) of the CF11 with and without CBD, with Their Respective Mean Error

	CF11 with CBD			CF11 without CBD		
probes	35 °C	42 °C	50 °C	35 °C	42 °C	50 °C
n-hexane	0.93 ± 0.01	0.68 ± 0.01	0.51 ± 0.01	0.84 ± 0.01	0.68 ± 0.03	0.47 ± 0.02
n-heptane	2.90 ± 0.01	2.06 ± 0.01	1.44 ± 0.01	2.67 ± 0.00	1.94 ± 0.03	1.31 ± 0.01
n-octane	8.94 ± 0.02	6.19 ± 0.02	4.13 ± 0.04	8.46 ± 0.02	5.73 ± 0.02	3.74 ± 0.02
n-decane	89.6 ± 0.4	57.0 ± 1.6	34.1 ± 0.7	84.3 ± 0.3	52.4 ± 0.3	31.4 ± 0.1
chloroform	1.98 ± 0.01	1.42 ± 0.00	0.99 ± 0.02	1.97 ± 0.01	1.40 ± 0.01	0.97 ± 0.01
THF	13.4 ± 0.4	8.81 ± 0.12	5.32 ± 0.20	15.9 ± 0.5	10.6 ± 0.2	6.56 ± 0.10
diethyl ether	3.01 ± 0.00	2.00 ± 0.01	1.30 ± 0.01	3.86 ± 0.03	2.55 ± 0.02	1.67 ± 0.02
acetone	10.0 ± 0.2	6.86 ± 0.06	4.26 ± 0.17	15.6 ± 0.3	10.9 ± 0.5	7.25 ± 0.19
ethyl acetate	20.7 ± 0.3	13.4 ± 0.4	8.31 ± 0.42	28.6 ± 1.1	19.7 ± 0.4	13.2 ± 0.5

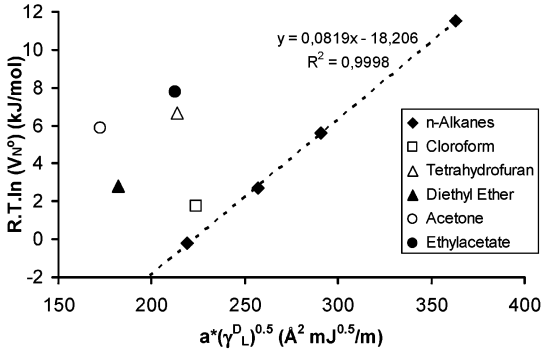

Figure 3. Surface free energy of adsorption vs $a\cdot(\gamma^D_L)^{0.5}$, for the CF11 with CBD, obtained at 35 °C. The equation presented corresponds to the *n*-alkanes reference line.

The specific retention volumes obtained with the several probes on the two columns are shown in Table 3. The values for the basic and amphoteric probes are reduced by 15 to 41%, in the presence of adsorbed CBD. The errors obtained in the retention volume calculations are below 5%, and thus the referred reduction is significant. It seems then that the basic and amphoteric have lower affinity for the fibers with adsorbed peptides. Another difference, although of lower magnitude, can be detected by comparing retention volumes: the neutral probes interact slightly stronger with the fibers with CBDs, suggesting that these reduce the surface polarity. This could be a consequence of the hydrophobic amino acids present in the CBDs being exposed and free to interact with the probes. Those amino acids would be expected to be interacting with the fibers, since they mediate the adsorption process. However, there is experimental evidence (not shown) that these peptides aggregate in aqueous solution, and that would explain why some of the adsorbed binding domains may expose their hydrophobic flat surface.

Similar graphs to Figure 3 were obtained for the fibers without CBD and for each temperature. The reference lines for the *n*-alkanes have a good correlation coefficient (above 0.999). The dispersive component of the surface free energy (γ^D_S)—slope of the reference line—is lower for higher temperature (Table 4); several authors,[16,22,25,26] also studying cellulosic materials, reported the same trend, explained as a consequence of an expansion of the fibers with temperature.[27] The γ^D_S value is influenced by changes in the fibers structure, according to the work by Balard et al.[28] The values presented here are in good agreement with the ones reported by other authors.[16,22,25,26]

Table 4. Values of the Dispersive Component of the Solid Surface (γ^D_S, mJ/m²) for the Temperatures and the Two Methods Used

method		35 °C	42 °C	50 °C
Schultz	CF11 with CBD	46.2 ± 4.0	43.4 ± 3.5	39.2 ± 4.1
	CF11 without CBD	47.0 ± 4.1	42.1 ± 5.7	39.4 ± 4.3
Doris and Gray	CF11 with CBD	47.3 ± 1.4	44.9 ± 0.5	41.1 ± 0.9
	CF11 without CBD	48.0 ± 0.5	43.5 ± 3.1	41.3 ± 1.6

In the present work, the γ^D_S values obtained with the different columns are similar, considering the errors associated. As remarked already, there is a difference between the retention volumes of neutral probes obtained with the two columns. This difference may have been annulled by the calculations associated to the γ^D_S value determination. The analysis of the retention volume values makes possible to detect differences that are no longer obvious when comparing the thermodynamic values calculated after the complex treatment of the experimental data. On the other hand, another explanation would be that CBDs increase the fibers surface area. This possibility will be exploited later in the present work. In such case, the retention volume for surfaces with similar apolar properties would be larger for the sample with larger surface area (CBD-treated), although the dispersive energy would be similar. Further study will focus on trying to demonstrate whether CBDs increase the dispersive character of the surfaces (by exposing hydrophobic amino acids) or whether an increase of surface area is the reason for these results. Since it has been reported two methods to calculate the value of γ^D_S, one by authors Schultz and Lavielle[13] and the other by Doris and Gray (as described by Bailey and Persaud[29]), they where both used in this work. The differences between the values obtained by those two methods may be explained considering that the latter assumes the alkanes area to be equal to the sum of as many CH_2 groups as the number of carbons. Indeed, each molecule has two methyl groups. Gutierrez et al.[30] refers that the Schultz method should be more reliable, because it does not use just one value of either γ^D_L or area.

Regarding $-\Delta G_{SP}$ (Table 5), the values obtained are higher for the amphoteric and basic probes. This result is expected because the cellulose fibers have −OH groups (responsible for the predominance of the C_2 peak in the XPS spectrum), which are electron acceptors, thus making the surface acidic.[31] The adsorbed CBD significantly reduce the interaction energies in the amphoteric and basic probes for the three temperatures studied. This result may be

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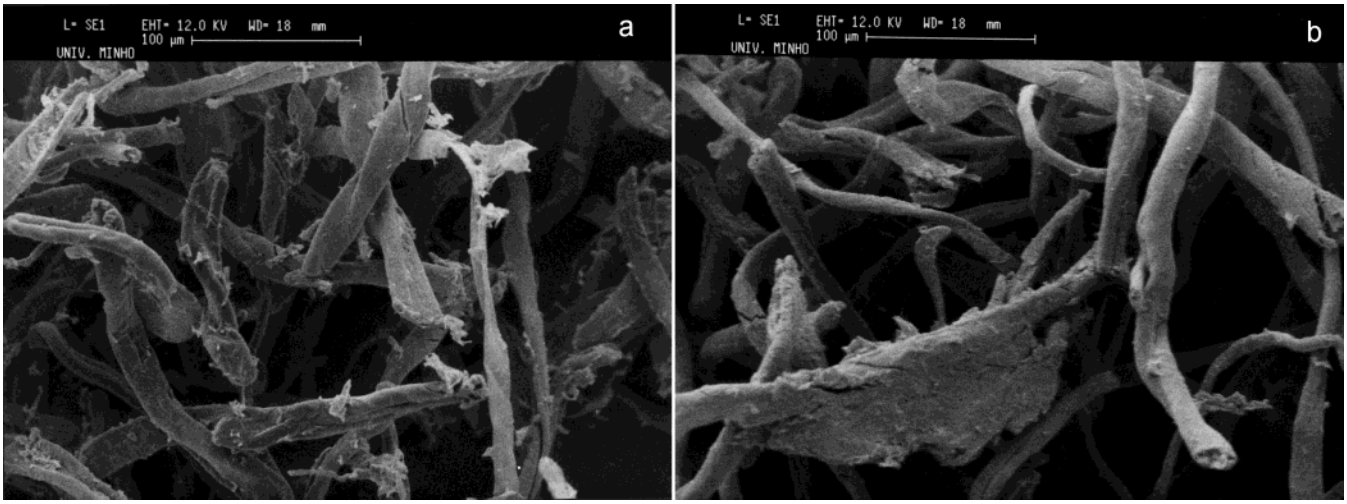

Figure 4. SEM images of CF11 fibers treated with (a) and without (b) CBD.

Table 5. Specific Surface Free Energy ($-\Delta G^{AB}_A$, kJ/mol) for the Interaction with Different Probes

	CF11 with CBD			CF11 without CBD		
probes	35 °C	42 °C	50 °C	35 °C	42 °C	50 °C
chloroform	1.63	1.57	1.40	1.85	1.59	1.62
THF	7.39	7.07	6.51	8.06	7.60	7.29
diethyl ether	6.12	5.77	5.26	7.02	6.41	6.17
acetone	9.97	9.67	9.01	11.4	10.9	10.6
ethyl acetate	8.57	8.23	7.72	9.64	9.26	9.16

explained by the occultation of hydroxyl groups on the fibers surface, thus lowering their acidic character.

The values for the acid and basic numbers (K_A and K_D, respectively) were also calculated but they are statistically not significant, mostly because of error propagation, which masks the differences observed in Table 5. Therefore, they are not presented in this work.

Xiao et al.[32] reported a reduction in the crystallinity index of a cellulose treated with a fungal endoglucanase CBD. In the present work, the crystallinity index (92.0% and 91.9%, with and without CBD, respectively) was not modified by CBDs, in agreement with the γ^D_S results; as a matter of fact, a reduction in crystallinity would probably imply a modification of the γ^D_S value. An interesting morphological modification was observed by SEM (Figure 4). Fiber agglomerates disappeared after the CBD treatment that simultaneously led to the appearance of small particles all over the fibers. Apparently, CBDs disrupt the aggregates that upon drying become spread on the surface of the larger fibers. Such a disintegration process may be associated to an increase in surface area, as suggested previously. This effect must be interpreted as an interfacial phenomenon. Indeed, IGC does not reveal a dramatic modification of the surface properties following the CBD treatment, besides the reduction in the acidic character. Then, the CBD treatment possibly reduces the interfiber interaction (deseggregating the fibers, as observed by SEM), by means of steric and hydrophobic effects.

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Conclusions

The IGC technique was used to detect surface energy modification following CBDs adsorption onto cellulose fibers. A reduction of the acid character of the fibers was detected and confirmed by ESCA analysis. No major modification of the dispersive component of the surface energy was detected, although the retention volume of neutral probes was slightly increased. This increase may be due to free hydrophobic amino acids present in the adsorbed CBDs or to an increase in the fibers surface area. CBDs appear to break down agglomerates presented between the fibers, but no modification of the crystallinity index was noticed. The modification of fiber properties by CBDs, detected in previous work, may also have an important contribution associated to steric or hydrophobic effects that were not assessed in these experiments.

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