Chapter 7

Cellulose-Binding Domains as a Tool for Paper Recycling

H. Pala[1], R. Pinto[1], M. Mota, A. P. Duarte[1,2], and F. M. Gama[1]

[1]Centro de Engenharia Biológica–IBQF, Universidade do Minho, Largo do Paco, 4719 Braga codex, Portugal (telephone: 351–253–604400; fax: 351 253)
[2]Current address: Departamento de Ciência e Tecnologia do Papel, Universidade da Beira Interior, 6201–001 Covilahã, Portugal

Treatment of secondary paper fibres with cellulose-binding domains allows for improvements of pulp drainability and of paper mechanical properties. The interfacial system fibre-water-fibre, and after drying, fibre-air-fibre, may be affected by the CBD treatment, influencing the pulp and paper technical properties. Inverse Gas Chromatography provides experimental evidence that support this hypothesis.

Introduction

Cellulose-binding domains (CBD) are functionally independent protein modules present in many cellulases. These modules are essential for the effectiveness of the enzymatic action, namely for the hydrolysis of insoluble cellulose and especially of the crystalline regions. In fact, whenever the CBD is removed, the hydrolysis of insoluble substrates by the catalytic domain alone proceeds at a much lower rate (1, 2, 3). The enzyme affinity and adsorption on cellulose is controlled by the binding core structure, topology and charge (4). The enzymatic modification of paper pulps depends on the CBD used. Indeed, a Kraft pulp treated with proteins from *Trichoderma reesei* - native cellobiohydrolase I and a recombinant cellobiohydrolase I with an endoglucanase I-binding domain - have different technical properties (3).

The binding domains retain their functionality when separated from the rest of the protein. It has been suggested that CBDs have the ability to disrupt the cellulose fibres surface (5, 6). This ability would explain the striking importance of CBDs in the hydrolysis of insoluble substrates: not only they allow for a concentration of the enzymes in the surface of the fibres, but also the swelling and fibre surface disruption favours the formation of new binding sites, facilitating the hydrolytic process. The CBD action on the fibre surface is certainly a topic that requires additional experimental studies.

To this point, research on the CBD properties has focused mainly on the CBD/cellulose interaction and on the binding/catalytic domains interdependence. In the present paper, the ability of family I CBD (obtained by proteolytic digestion of *Trichoderma reseei* cellulases) to modify the technical properties of a secondary pulp for paperboard production is demonstrated. Paper fibres upgrading with cellulases has been attempted since over the last 20 years. As shown in previous work (7, 8), enzymatic modification of fibres is not simply a hydrolytic process. In fact, the modification of the interfacial properties may be a rather important aspect. To characterise the modification of interfacial properties by CBDs, Inverse Gas Chromatography was used in the present work.

Methods and Materials

Cellulose-binding domains

The cellulose-binding domains (CBD) preparation was obtained by ultrafiltration after proteolytic digestion of Celluclast 1.5L, according to the protocol described in Lemos *et al.* (9). The final CBD solution did not show cellulolytic activity (9); protein quantification was done by the Bradford method (10).

Cellulose powder: Whatman CF-11 was used in the Inverse Gas Chromatography experiments.

Paper pulp: The pulp used in this study was obtained after disintegration of old paperboard containers, representing a mixture of 60% Kraft paper, 20% fluting and 20% test liner. It was kindly supplied by the company *Portucel Viana.*

CBD treatment of paper pulps

Paper pulps (a mass of wet pulp equivalent to 70 g of oven dried (o.d.) pulp) were disintegrated with a blender in sodium citrate buffer 0.05 M, pH 5.0, for 10 minutes. The CBD solution (representing 10% of the total reaction volume to assure a good dispersion) was then added to the mixture and allowed to react for 30 min, with continuous slow mixing (3% consistency, at 50°C). Then, the fibres were recovered by filtration. The filtrate was forced through the fibre cake, in order to avoid the loss of shorter fibres. Reducing sugars in the filtrate were measured using the DNS method (11). The pulp was treated with 1.1 mg protein/ g o.d. pulp. Control assays were executed in the absence of CBD. Each assay, with and without CBDs, was performed twice with good reproducibility.

Preparation of paper sheets

Paper sheets (60 g/m^2) were prepared using an Auto Dinamic Sheet Former (DSF), manufactured by Noram – Lorentzen & Wettre.

Pulp and Paper Testing

Determination of the pulp and paper properties followed the usual standard procedures: drainage rate (ISO 5267/1), burst (ISO 2758), tensile strength (ISO 1924/2), tear (ISO 1974), sheet density (ISO 534) and permeability to air flow (ISO 5636/3). Test pieces for paper characterisation were obtained from the middle region of the DSF sheets; a four-sheet set (0.198 m^2 each) was available for each experimental condition. The coefficients of variation (drainage, tensile, burst and tear) were less than 1%.

Inverse Gas Chromatography

In gas chromatography, the retention volume of a volatile substance depends on its interaction with the stationary phase. In Inverse Gas Chromatography (IGC), the solid adsorbent to be characterised is the stationary phase, while known probe molecules are used as volatiles. When the measurements are carried out at infinite dilution of the solutes or at zero surface coverage of the adsorbent, then the net retention volume V exclusively derive from the interaction between adsorbent and adsorbate. The net retention volume is given as: $V=F(t_r-t_0)$, where F is the corrected flow rate of carrier gas, t_r is the retention time of the respective solute and t_0 is the retention time of the marker (methane). The complete thermodynamic characterisation of the adsorbent solid phase is

possible, by using the appropriate probes and measuring the retention volume at different temperatures, as described elsewhere (12, 13). When aiming at comparing the properties of different solids, the direct comparison of the retention volumes, at a defined temperature, provides an accurate measure of the relative affinity of the materials for each probe.

In this work, the effect of CBDs on the surface properties of a model cellulose powder (Whatman CF-11) was studied. A suspension of cellulose (total cellulose weight of 10 g) was prepared in sodium acetate buffer 0.05 M, pH 5.0. The CBD solution (representing a minimum of 10% of the total reaction volume, to assure a good dispersion) was then added to the mixture (final concentration: 3% $w_{cellulose}/v_{buffer}$; 0.2% $w_{protein}/w_{cellulose}$) and allowed to react for 30 min, with continuous slow mixing, at 50°C. The mixture was then centrifuged at 4000 rpm. The supernatant was removed, the fibres were washed with distilled water (300 ml) and again centrifuged (method 1). In another experiment, the same procedure in the preparation of the samples was carried out, with the exception that the fibres were washed twice with 400 ml of distilled water (method 2). For both methods, the respective control was prepared, using a buffer solution without CBDs. The obtained material was packed in a Chrompack stainless steel column of 1 m length and 4 mm internal diameter. About 5 grams were packed in each column. The exact weight was recorded and used to correct the values of retention volume. The non-processed Whatman CF-11 fibres were also analysed.

Chromatographic measurements at infinite dilution were carried out with a Chrompack CP9001 gas chromatograph equipped with a flame ionisation detector. The carrier gas was helium. The sample was injected with a Hamilton Gastight 1750SL syringe, and its concentration was adjusted such that the minimum attenuation was used in the determination of the retention time, ensuring practically infinite dilution. The retention volume of each probe, V, was calculated as the average of at least 5 values.

Results and Discussion

Table I summarises the properties of the paper sheets and the effect of the cellulose-binding domains (CBD) treatment. A positive effect on both the pulp and paper properties is detected. In fact, both drainage and strength (specially tensile and burst) are improved by the CBD treatment. The paper sheets do not show any major change in the density or permeability parameters. This trend contrasts with the effect typically obtained when enzymes are applied for fibre modification (7); although improving drainage to a large extent, the enzymatic hydrolysis normally worsens the paper mechanical properties. Furthermore, as expected, no solubilisation was detected after the pulp treatment. Indeed, the possibility of excessive (hydrolytic) modification of the fibres, with reductions both in paper yield and fibre quality, is not expected to be an issue when using CBDs.

Table I. Effect of the CBD Application on the Properties of Pulp and Paper

	Drainage (°SR)	Tensile (Nm/g)*		Burst (KPam²/g)	Tear (mNm²/g)*		Permeability to air (ml/min)
		MD	TD		MD	TD	
Control	43	30.3	11.6	1.0	3.6	6.8	2714
CBD assay	35	36.5	12.6	1.1	3.7	5.8	2831

* MD. machine direction; TD, transversal direction.

The possible application of CBD in recycling has been previously proposed (7). However, the present work provides further evidence that the non-hydrolytic peptides may be a valuable additive for the paper industry. In the previous work, paper strength was measured in handsheets where, as a consequence of random deposition, fibres do not present a determined orientation. By contrast, the dynamic sheet former (DSF) used in this work allows for an optimal fibre orientation during sheet formation. As in the paper-machine, the obtained paper sheets can be characterised under two directions (Figure 1): along with, and transversally to the fibres orientation in the paper sheet (machine-direction, MD; and transversal-direction, TD). The results show that the magnitude of the modification achieved with the CBDs is greater in the "stronger" direction of the paper sheet. The effectiveness of CBD may be associated to a better fibre alignment (and increased interaction sites between fibres), which would explain the increased paper resistance to tensile forces in the machine-direction. Resistance improvement along the transversal direction is inferior (20% MD *versus* 9% TD) because although fibre alignment provides an increased number of connections, the inter-fibre length connection along this direction is lower.

A relevant question remains unanswered: why do the non-hydrolytic CBDs allow for both drainage and strength improvements? In our opinion, CBD modify the fibres interfacial properties and thereby the pulp and paper properties. The high fibre-affinity of the peptide and its surface activity may be responsible for the fibre surface modification. In fact, an increased strength of the fibre-water interaction has been observed in paper fibres and powdered celluloses treated with cellulases (8, 14). The referred results show that the adsorbed enzymes increase the fibre water-affinity, leading to a stronger water adhesion. This effect may be responsible for the fibre stabilisation in aqueous suspension, thus avoiding the formation of preferential draining channels and leading to a more homogeneous paper sheet. Upon drying, the inter-fibre interaction is again possible (Figure 2). CBD and enzymes should modify the interfacial properties in a similar way. However, the hydrolytic activity is often detrimental to the paper strength, owing to the reduction in the intrinsic fibre strength, while a positive effect is obtained with CBD. The catalytic activity, and the much higher
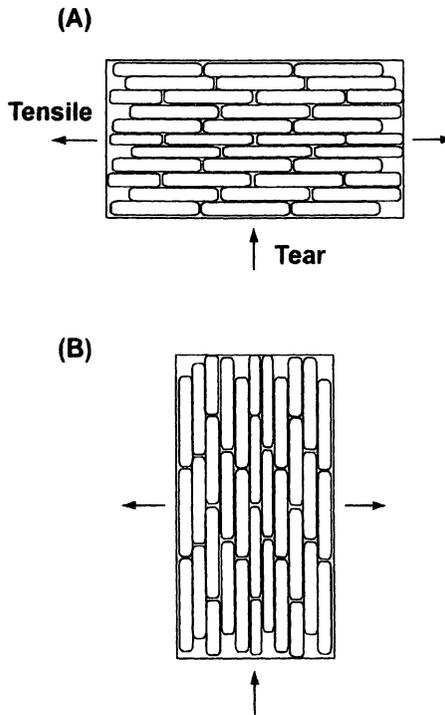
(A)

Tensile

Tear

(B)

Figure 1. Paper direction influencing paper strength

Fibre alignment in paper sheets increases the number of interfiber contact sites thus improving paper strength. When tensile is measured along the paper MD (A), the number of interfibre bounds opposing the applied strength is higher than when it is determined along the paper TD (B), thus explaining the increased paper resistance to tensile in the former situation. As in respect to tear, paper TD (A) offers a higher resistance to the applied force because in this direction fibres themselves have to be broken.

molecular weight of glycosyl hydrolases (*versus* CBD), may contribute for the different effects in the paper properties. More data on the use of CBD for pulp and paper modifications is necessary in order to support these hypotheses.

It must be remarked, with regard to Figure 2, that the interaction between adjacent fibres is critically affected by the presence of water. The Hamacker constant, a measure of van der Waals forces between surfaces (15), is much higher for condensed phases interacting across air, as opposed to the interaction across water. In the case of cellulose, the Hamacker constants, determined according to the Lifshitz theory are 5.8×10^{-20} J (interaction across air) and 0.8×10^{-20} J (interaction across water) (16). Therefore, the fibre stabilising effect in aqueous suspension (a consequence of steric and hydration phenomena) no longer applies upon fibre dehydration, when the attracting van der Waals forces between the fibres are higher and hydration effects are not present. The CBD activity may be interpreted as a refining-like process, according to Milichovsky molecular interpretation of the process (17). Indeed, it has been recently shown (6) that CBD may lead to fibre disruption and crystallinity reduction, directly affecting the fibres hydration layers.

The characterisation of a model cellulose using IGC shows that washing the fibres modifies significantly their surface properties, possibly due to the removal of ions or other adsorbed chemicals (Table II, CF-11 *versus* Control 1 and 2).

The differences between the two controls suggest that washing the fibre, as carried out by method 1 (washed once with 300 ml of distilled water), is not sufficient. Indeed, the retention volumes obtained for Control 1 lie between those of the non-treated fibres CF-11 and Control 2, prepared by more thoroughly washing the fibres with distilled water (2x400ml). Comparing the Controls with the respective CBD assays suggest the same general trend. Interestingly, the treatment with CBDs does not affect the dispersive properties of the surface, since the retention volume of neutral probes is not affected (Table II, Control *versus* CBD). The interaction with an acidic probe, chloroform, is also not affected. It seems that the cellulose surface has essentially an acidic character, since it interacts preferentially with basic and amphoteric probes, which have larger retention volumes. The presence of CBDs contributes to a reduction of this acidic character, as can be concluded from the significant reduction in the retention volume detected for tetrahydrofuran, dietilic eter, acetone and ethyl acetate. The major modification CBDs introduce in the surface properties seems to be a reduction in the concentration of acidic groups, presumably because a part of the cellulosic surface is not accessible following the CBD treatment. Apparently, the stabilisation of the fibres in aqueous suspension is therefore a consequence of steric and hydration effects, as suggested elsewhere (8), and not
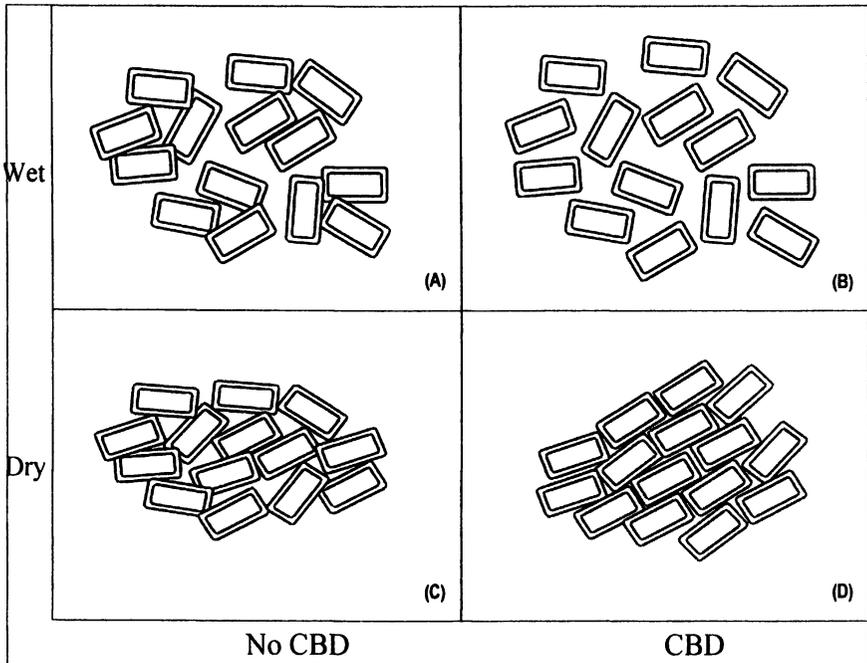
Figure 2. Interfacial fibre modification by enzyme adsorption

(A) Fibres in suspension, with no CBD. The fibres hydrophobic character causes aggregation. The presence of the aggregates affects sheet structure, due to the occurrence of preferential dewatering areas during sheet formation. The fibres arrangement in the paper sheet (C) does not fallow an optimal orientation pattern, thus affecting the product final characteristics. (B) Fibres in suspension, with CBD. The stabilised fibres allow water to flow freely, leading to a better-formed sheet: the fibres orient themselves towards the same direction, increasing the surfaces contact for bonding (D).

Table II: Retention Volume of the Probes Used in the Inverse Gas Chromatography Experiments

Probe	V_n (ml)				
	CFI1	Control 2*	CBD 2*	Control 1*	CBD 1*
Hexan - n	0.231±0.009	0.604±0.014	0.471±0.019	0.465±0.017	0.509±0.006
Heptan - n	0.76±0.012	1.745±0.015	1.439±0.015	1.309±0.007	1.438±0.007
Octan - n	2.061±0.025	5.030±0.021	4.123±0.028	3.737±0.016	4.127±0.038
Decan - n	16.277±0.025	42.652±0.105	35.205±0.119	31.391±0.045	34.070±0.706
Chloroform - a	0.453±0.022	1.343±0.016	1.051±0.025	0.974±0.012	0.986±0.019
THF - b	2.894±0.041	10.121±0.113	7.502±0.026	6.556±0.102	5.324±0.201
Dietilic eter - b	0.713±0.015	2.520±0.031	1.982±0.021	1.674±0.021	1.297±0.012
Acetone - p	3.635±0.068	11.836±0.297	7.800±0.178	7.253±0.190	4.255±0.166
Ethyl acetate - p	5.296±0.266	21.364±0.476	14.722±0.211	13.218±0.538	8.313±0.424

* Samples prepared according to method 1 or 2 (material and methods section); n-neutral; a-acidic; b-basic; p-amphoteric

of the increase of surface polarity. A comprehensive thermodynamic characterisation of the fibres interface will provide a better understanding of the complex phenomena associated to the CBDs modification of cellulosic fibres.

Conclusions

Cellulose-binding domains seem to be a performant additive for paper recycling. They probably share some of the good enzyme properties: hydration and slight surface disruption of the fibres (in a way similar to refining), without its drawbacks: fibre solubilization, intrinsic fibre strength reduction. The hydration and stabilisation of the fibres may lead to better paper sheet formation, resulting in improved paper resistance. Enzymes may have a similar effect, in some cases, but hydrolysis of the fibres surface is, probably, mainly detrimental (7). Inverse Gas Chromatography confirms that CBDs modify the interfacial properties of a model cellulose. The major effect of CBDs adsorption on the surface properties of Whatman fibres is the reduction of the acidic character, with maintenance of the dispersive energy.

Acknowledgements

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