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Interactions of cotton with CBD peptides

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Abstract

The binding of genetically engineered Family II cellulose binding domains (CBDs) of *Cellumonas fimi* cellulases to cotton fabrics was studied and possible textile applications were investigated. Family II CBDs bound to cotton cellulose increased dye affinity, especially for acid dyes, but with very poor washing fastness. Ironing of the protein bound fabrics before dyeing increases dye affinity, which is probably due to protein denaturation and thus increases exposition of ionic groups. For desorption of CBD proteins from the cotton fabric, high levels of mechanical agitation and alkaline conditions (pH >7) are necessary. Binding of Family II CBDs to cellulose releases fine particles, whereas migration and desorption did not. Long time storage of cotton fabrics with adsorbed CBD proteins did not cause changes in their physical properties and did not damage cotton cellulose. The presence of water on the surface of cellulose was found to be essential for the interfabric migration of Family II CBDs. The double binding cellulose domain binds strongly on cotton and their interfabric migration is smaller.

Keywords: Cellulose binding domain (CBD); Cellulases; Cotton; Adsorption

1. Introduction

Textiles and detergents are worldwide the largest markets for cellulase enzymes, since the beginning of the 1990s. The fashionable stone washed look of Denim garments and other processes that improve fabric appearance by removing fuzz fibres and pills or deliver softening benefits are all well known in the area of "cellulase finishing" [1]. Increased use is also being made of cellulases in domestic fabric washing products where they are claimed to aid detergency and to clean fibre surfaces, improving appearance, and color brightness [1,2]. All these fabric benefits are a result of the hydrolytic action of a soluble enzyme on an insoluble substrate like cellulose and it can be expected to find in cellulase enzymes a structural-functional relationship. In fact, most cellulases have two functionally distinct domains [2]: A catalytic domain and a substrate binding domain or cellulose binding domain (CBD). Both domains are linked by a glycosylated linker peptide.

The catalytic domain has an active site in the shape of a tunnel or a cleft where the hydrolytic reaction takes place. Based on amino acid sequences comparisons and hydrophobic cluster analysis, 11 families have been proposed to group all known cellulase catalytic domains [3]. The linker is a shorter peptide and seems to have an important role on the inter flexibility between the catalytic domain and the CBD [4]. The presence of a CBD is essential for the degradation of crystalline cellulose like cotton cellulose and it is believed that the presence of a cellulose binding domain within the enzyme structure increases the concentration of the enzyme nearby the insoluble cellulose and enhances the catalysis [5,6].

The cellulose binding domain is a peptide, whose length varies depending on its origin between 33 to 240 amino acids. Based on sequence homologies they are classified into several different families. Several 3-dimensional structures of CBDs (Families I to IV) have been solved and are available from Brookhaven Protein Data Bank [6,7]. Family I CBDs (~36 amino acids) are just found in fungal cellulases commonly used for textile and detergent applications (either acid or neutral cellulases). These shorter binding domains were found to bind reversibly to cellulose [8], whereas Family II CBDs seem to bind irreversibly to cellulose [6,9]. Family II CBDs are found in bacterial cellu-

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Table 1
Protein size and protein amount adsorbed on the fabrics

Proteins (*Cellumonas fimi*—with CBDs of Family II)	MW (kDa)	Protein bound (mg/g fabric)
CenA D392A—Inactive endoglucanase A	43.8	3.6
DBD392—Inactive endoglucanase A with a extra added CBD of Family II (inactive catalytic domain with 2 CBDs)	56.6	2.9
CBDCenA—Cellulose binding domain of endoglucanase A	11.3	4.5

lases having around 110 amino acids. Other families have even bigger binding domains. The presence of tyrosine in the planar strip of family I CBDs [10] and tryptophan in Family II CBDs [11] seems to play an important role during the binding to cellulose [7]. It is believed that CBDs face the (0.2.0) crystallographic planes located at the corners of a perfect cellulose crystal where the surfaces of a glucopyranoside ring are fully exposed making them available for hydrophobic interaction with aromatic rings like side chains of tyrosine or tryptophan [7]. Family II CBDs were reported to disrupt the surface of cotton fibers and release fine particles [5,6]. This disrupting activity was not found on CBDs of other families [6]. These disruptive forces findings might have some important implications in the detergent industry, concerning to soil release processes.

The use of genetic techniques makes CBD peptides now available in industrial quantities [9]. The binding properties of Family II CBDs have a potential for new finishing effects on cotton; therefore, it is desirable to know about the behavior of cotton fabrics adsorbed with these products.

2. Materials and methods

2.1. Preparation of the protein adsorbed fabrics

Cotton fabrics were incubated with clarified cell extracts of *Escherichia coli* (see details in [9] and [11] for plasmid constructions of CenA D392A, DBD392, CBDCenA from *Cellumonas fimi*) diluted in 0.05 M sodium phosphate buffer, pH 7. Incubation was carried out in LabMathis pots at 35°C during 30 min and with gentile rotation. Afterwards the treated fabrics were washed 10× under tap water and line dried. Protein on the fabric was measured by modified Lowry method [12]. Protein size and protein amount adsorbed to the fabrics are displayed in Table 1. In the remaining liquor of CBDCenA a further adsorption step was carried out with a fresh fabric. The process was repeated until we had 5 fabrics with different concentrations (see diagram of Fig. 1 for concentrations). The reference fabric was just washed with buffer (a negative control were done with a cotton fabric incubated in similar conditions as above with cell extracts of *E. coli* harboring the vector alone, and just traces of protein were found in the fabrics after the 10 washes).

2.2. Dyeing, washing, and desorption tests

The CBDCenA adsorbed fabrics were dyed with 2% solution of Coomassie Brilliant Blue G (Merck; Acid Blue 90, C.I. 42655). The washing of the dyed fabrics with neutral soap was performed according to ISO 105-CO3-1978 standart (60°C and 60 min). Washing with water was done under the same conditions, but just with water and no chemicals. Desorption tests were performed in a shaker bath (over night at 25°C) Ahiba Spectradye and in a Rotawash machine (1 h, 50°C) with acetate 0.1 M (pH 5), phosphate 0.01 M (pH 7) and carbonate 0.005 M (pH 10) buffers. Protein on the fabric and in solution were measured by modified Lowry method [12]. K/S values (color staining levels) at 600 nm were measured with an ACS Chroma Color Reflectance Spectrometer. Ironed fabrics were ironed 5× on each side with a common household iron adjusted to cotton ironing temperature.

2.3. Fluorescence measurements

Fluorescence spectra of protein adsorbed cotton fabrics (untreated, dry and wet ironed) were measured with an excitation wavelength λ_{exc} of 296 nm on a Perkin–Elmer Spectrophotometer with a reflectance facility.

2.4. Interfabric migration of experiments

2.4.1. In solution

A protein bound fabric piece (5 × 5 cm) was incubated with 20 ml of distilled water with a witness fabric (no protein bound and same size) over night at room temperature in a shaker bath. Protein in solution and on the fabrics were analyzed by the modified Lowry method [12].

2.4.2. By friction

A dry fabric bound protein was rubbed (10 cycles forward/backward under certain force in a Crockmeter, James Heal Co, Ltd) against other fabric (witness) with no protein bound. Dry and wet witnesses were used. The increase of the protein content in the rubbed area of the witness fabric was measured by modified Lowry method [12].

2.5. Reducing ends on fabric, strength loss, and reversibility of the protein binding to the fabric in aqueous solution

Fabric reducing ends, fabric strength loss, bound protein, and reversibility of binding were measured on the protein bound and dried fabric 5 days and 2 months after the adsorption experiments. Reducing ends and strength loss were measured as described before [13]. To measure the reversibility of the protein binding to the fabric 200 mg of

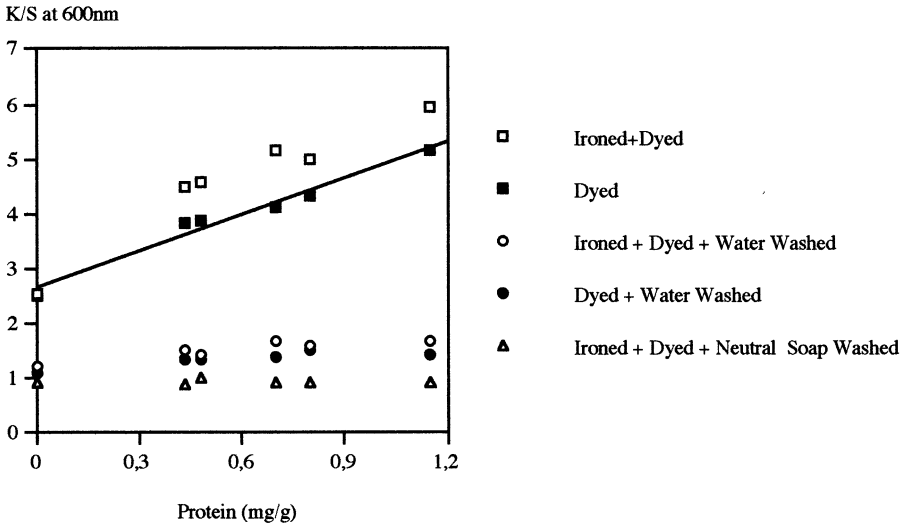

Fig. 1. Staining levels (K/S) of fabrics adsorbed with CBD-CenA and dyed with Coomassie Brilliant Blue G with and without previous ironing and before and after washing.

the fabric were incubated overnight in a shaker bath in 10 ml of water. Protein on the fabric and in solution were measured by modified Lowry method [12].

3. Results and discussion

3.1. Dyeing, washing, and desorption tests

The dye affinity for a fabric "coated" with a protein may be expected to increase, because at milder pHs (pH 5–8) cellulose has a low amount of ionic groups present. Actually, the adsorption of cotton fabrics with CBDCenA resulted in higher staining levels after dying (see Fig. 1). A linear relationship between the amount of protein (CBD-CenA, see Table 1 for details) bound to the fabric and the staining levels (K/S at 600 nm) of Coomassie Brilliant Blue G was found (see Fig. 1). Coomassie Brilliant Blue G is an acid dye (C.I. Acid Blue 90) with an increased affinity for proteins. Similar observations were made before with the Indigo staining of cotton [12], that enabled a better understanding of backstaining during cellulase washing of Denim. We also observed an increased dye affinity of protein bound fabrics for direct dyes. These results would be interesting if protein binding would be stable under normal conditions of processing and wearing of cotton fabrics. Therefore, CBDCenA bound fabrics were dyed (with and without former ironing) and washed under usual testing conditions. Results are displayed in Fig. 1.

Ironing of the fabrics before dying resulted in a darker shade and, thus, in higher K/S values. Ironing leads probably to denaturation of the bound CBD protein. More ionic groups become exposed and are thus available for dye fixation. However, no shift of the maximum adsorption (at 339 nm) of tryptophan fluorescence for the was observed of CBD bound fabric. Compared to the results with an entire cellulase protein with CBD, linker and catalytic domain the effect of ironing on the increase in the staining levels is small [14]. This is not surprising because the amino acid chain is much shorter and the probability of additional hydrophobic interactions with cellulose caused by unfolded tryptophan residues is smaller. Another possible explanation for the fluorescence results is that most of the tryptophan residues are already involved in interactions with the substrate [7,11], so that protein denaturation does not change significantly their neighborhood conditions.

Washing fastness of the dyed fabrics against neutral soap at 60°C was very poor (see Fig. 1). Almost all of the dye was removed, colour intensity was decreased at least by 65% and no differences in K/S between fabrics adsorbed with different amounts of CBDCenA were found after washing. Fabric witnesses did not show any dye staining. Even by washing with water at 60°C K/S values were reduced to a large degree, but staining levels were higher than after washing with neutral soap. Small differences in K/S could be still detected with different amounts protein previously adsorbed in the fabric. Fabric witnesses became slightly stained. The differences in staining of the fabric witnesses are due to the formation of micelles by neutral soap, and their solubilization of the dye. Micelles keep the dye in solution and avoid its redeposition on the witness. On the other hand, the staining of the witnesses may be due to migration or desorption and re-adsorption of the adsorbed protein. In general, ironing of the fabrics before dyeing resulted in slightly higher staining levels after washing with water. This can be explained by the formation of additional hydrophobic interactions between fabric and protein. More hydrophobic interactions mean stronger binding and more resistance against the dissolving forces of water.

The reversibility of the adsorption of CBDCenA to cot-

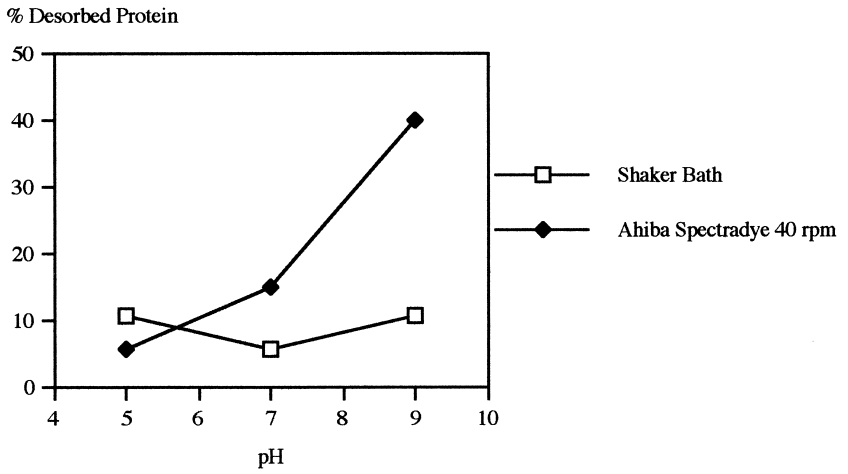

Fig. 2. Desorption of CBDCenA (in percent of the initially bound protein) from cotton fabric at different pHs and different levels of mechanical agitation.

ton was investigated with acid (pH 5), neutral (pH 7), and alkaline (pH 10) buffered elution solutions under different levels of mechanical action (Fig. 2). At low mechanical agitation on a shaker, little desorption was observed after 26 h. However, desorption values were near the limit of the sensibility of the method because very low initial amounts of protein were present. At higher levels of mechanical agitation (Ahiba, Rotawash) and at higher pHs (>7) up to 40% of protein were desorbed.

We also analyzed the desorption of proteins from ironed fabrics at pH 7. At both levels of mechanical agitation no significant desorption was noticed. If the protein is denatured, then it could be expected that desorption would be easier, but this was not the case. Instead of this, the protein seemed to be slightly stronger bound. This may be due to increased hydrophobic interactions of the denatured protein with cellulose, like it was suggested before [14].

Even after storing the fabrics adsorbed with CBDCenA over 6 months, no increase in soluble reducing sugars and, thus, no hydrolytic activity was detected. This coincides with the findings of Din et al. [5].

Previous results [9,10] reported, that bacterial CBDs of *Cellumonas fimi* (Family II) could only be eluted from different cellulose matrices with denaturing agents, but not with usual buffer solutions from pH 2.2 to pH 10. Confirming the results of Reinikainen and Ong [9,10], where little mechanical action was applied, our results from shaker experiments did not show any desorption of CBDCenA. However, at higher levels of mechanical action and under neutral or alkaline washing conditions, a considerable desorption of CBDs of Family II was observed (Fig. 2).

During the desorption experiments with the CBDCenA even with the synergistic effects of mechanical agitation no particle release was observed. However, during adsorption to cotton cellulose some turbidity was found. In coincidence with the results of Din et al. [5], CBDCenA adsorption to cellulose seems to release fine cellulose particles, whereas rewetting of the CBD bound fabrics, as in desorption and interfabric migration experiments, does not release detectable particles.

3.2. Protein size and structure effects

The size of the used proteins and the amount adsorbed on the fabrics are shown in Table 1. From results in Table 2 we see, that the presence of water seems to be the most important factor for interfabric migration of proteins. Whereas depending on the type of CBD in solution up to 30% and by wet friction up to 10% of the protein migrated to the witness fabric, no protein transfer between fabrics was observed by dry friction. In aqueous solution migration seems to depend on the number of CBD units in the protein. The migration of the double cellulose binding domain DBD392 was just 50% of the migration of the proteins containing one CBD. This can be explained by the formation of more hydrophobic interactions with the cellulose and thus a stronger binding of the protein. Migration by wet friction seems to be dependent on the protein size. Supposing, that on a bigger protein more surface area is available, friction forces can attack more efficiently and more protein can be removed. Recent differential scanning microcalorimetry results [6] suggest that binding to crystalline cellulose is associated to dehydration of the contact surface by the CBD. Our results confirm the important role of water during binding and migration. The fact that the protein does not go into solution (in the shaker

Table 2
Interfabric migration of CBD-protein in percentage of initially bound protein

Protein	Migration in solution (%)[a]	Migration by wet friction (%)	Migration by dry friction (%)
CenA D392A	30	10	0
DBD392	15	8	0
CBDCenA	33	3	0

[a] No protein found in solution.

Table 3
Properties of the fabrics measured after 5 and 60 days of storing

Protein	Cotton reducing power (mg glucose/g fabric) after		Breaking load (N) after	
	5 days (±0.05)	60 days	5 days (±20)	60 days
Control	0.33	0.31	273	277
CenA D392A	0.39	0.37	276	292
DBD392	0.37	0.32	274	294
CBDCenA	0.38	0.34	280	300

bath) indicates that migration might be caused by the contact of a CBD adsorbed surface with another wet cellulose surface. These phenomena is not clear and needs further investigation.

After 5 and 60 days of storage the properties of the CBD adsorbed cotton fabrics were not significantly changed (Table 3). Apparently the investigated proteins are not damaging cotton fibers by staying adsorbed to them for a longer time. The disruptive behavior found during binding of CBDs of Family II [5] is apparently not detectable after stable binding during 2 months.

4. Conclusions

As proteins Family II CBDs, when they are bound to cotton cellulose, increased the dye affinity of cotton fabrics, especially for acid dyes that have a high affinity for proteins. This can be interesting for the development of new dying procedures for cellulosic fibres. However, washing fastness under alkaline washing conditions was very poor for all cases. For possible applications, new methods of fixation, maybe by chemical binding or cross-linking, have to be investigated. Ironing of the protein bound fabrics increased dye affinity, which is probably due to the denaturation of the protein and an increased exposition of ionic groups to the surface.

We proved that the main reason for the poor washing fastness is the reversibility of the cellulose binding of Family II CBDs under washing conditions at higher pHs and high levels of mechanical agitation, as applied in common washing machines. Without agitation, Family II CBDs bound to a cotton fabric were found to migrate in aqueous solution to a considerable degree to other cotton fabrics. The presence of one more CBD in the protein reduced migration by 50%. We assume that this is directly proportional to the quantity of hydrophobic interactions formed between the protein and the cellulose.

During the adsorption to cellulose Family II CBDs release fine cellulose particles, whereas during further wetting of the fabrics once dried no such behavior was observed. Reasons are quite unclear and further investigation is needed.

Family II CBDs stay adsorbed on cellulose surfaces relatively stable for large periods without damaging cellulose.

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