Influence of Cellulases on Indigo Backstaining

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ABSTRACT

We have found that increasing concentrations of fungal cellulases on a fabric decrease indigo staining levels. Deletion of the cellulose binding domains (CBD) from either bacterial or fungal cellulases decreases indigo staining levels and generally causes less backstaining than the entire enzyme. Increasing the concentration of cellulases with a CBD of family I on fabric decreases indigo staining, whereas increasing the concentration of cellulase with a CBD of family II has no effect on staining. After-washing experiments of indigo-stained cotton fabrics show that it is easier to remove indigo adsorbed on cellulase adsorbed onto cotton than indigo directly adsorbed onto cotton.

The redeposition of indigo dye back onto denim fabric is known to occur during cellulase washing. There have been various attempts to reduce the effect of backstaining: adding proteases in denim finishing with cellulases was reported to achieve an improvement in the contrast due to a reduction in dye redeposition [6], and comparisons of cellulases and their effects on backstaining were made on the basis of equal activity units and equal amounts of adsorbed protein [5] or equal quantities of liquid commercial enzymes [7]. Opinions vary about the assumption that the degree of backstaining caused by one cellulase is related to its activity on the substrate [7] and the assumption that the nature and amount of the protein adsorbed on the fabric is the decisive factor [3, 5, 7]. In a first attempt to understand the mechanism of indigo backstaining [5], we found that the major reasons for backstaining are cellulase adsorption on the fabric and therefore binding of indigo to the cellulases bound to cellulose as well the nature of the cellulolytic protein itself. Indigo showed a lower affinity to a neutral cellulase of *Humicola insolens* than to an acid one of *Trichoderma reesei* [3, 5]. Furthermore, we found that the particle size of the indigo dye agglomerates was influenced by cellulase origin and concen-

tration [3]: *Humicola insolens* crude proteins seem to agglomerate the dye, while *Trichoderma reesei* crude proteins will disperse and reduce the size of indigo particles. All those studies [3, 5, 6, 7] confirm what is empirically known: that neutral cellulases enhance lower levels of backstaining than acid cellulases.

Adsorption by insoluble cellulosic fibers is a fundamental step in the hydrolytic action of the enzymes. Cellulases are known to consist of two structurally different domains: a catalytic domain and a cellulose-binding domain (CBD), which are linked by a peptide that influences their mobility to each other. According to their amino acid sequences, CBDs, which play an important role in binding to cellulose substrates, have been divided into several families [1]. Cellulases commonly used in the denim finishing industry have CBDs from family I (30–36 amino acids, *i.e.*, fungal cellulases from *Trichoderma reesei* and *Humicola insolens*), whereas CBDs of *Cellulomonas fimi* bacteria belong to family II (103–146 amino acids) [5].

Klahorst *et al.* [7] reported that denim finishing with acid cellulases, which are more aggressive on cotton cellulose, requires half the time or five to ten times less enzyme quantity to give the same level of abrasion (color removal) than with neutral cellulases. They also found that backstaining from treatment with acid cellulases depends more on washing time than on enzyme concen-

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tration, and that backstaining is primarily related to the degree of enzyme activity at a given pH.

In this work, we report a study of the backstaining phenomenon, focusing on the adsorption of indigo onto cotton fabric with and without pre-adsorbed cellulases. We also study the effects of the CBD family type present in cellulase enzymes on backstaining. Finally, we investigate after-washing steps to reduce backstaining, looking at the effects of mechanical agitation, protease treatment, and detergent.

Experimental

The washing experiments with indigo alone (pure indigo, BASF, lot number 7976670) were done on 2 g of a pre-washed (distilled water, 50°C, 20 minutes) cotton fabric in 50 ml of a buffer solution (pH 5, 0.01M acetic acid/NaOH or pH 7, 0.01M $\text{KH}_2\text{PO}_4/\text{NaOH}$) at 25°C. After 20 minutes, different concentrations (0, 6.25, 12.5, 25, 50, 125, 250, and 500 mg indigo/g fabric) of indigo powder were added and the fabric was washed for another 20 minutes (25°C, liquor ratio of 1:13.75, 40 rpm in a Rotowash). Fabrics were rinsed thoroughly, dried at 50°C, and the *K/S* at 650 nm was measured (ACS II Spectrometer).

The washing experiments testing the effect of cellulase concentration involved 2.5 g mercerized and previously washed (10 minutes, 50°C, 40 rpm in a Rotowash) cotton fabric for 20 minutes (Rotowash 40 rpm) in 80 ml of buffered (pH 4.85, 0.1M acetic acid/NaOH) and chilled (8°C) solutions of different concentrations of a total crude mixture of the fungi *Trichoderma reesei* (0, 0.5, 1, 2, 4, 6, 10 mg enzyme/g fabric). For the analysis of adsorbed protein, a sample of 0.5 g fabric was taken for each case. Indigo (50 and 500 mg/g fabric) was added in the previous bath and washed for another 20 minutes at 20°C (Rotowash, 40 rpm). Finally fabrics were washed for 20 minutes with distilled water (shaker at 25°C), rinsed, and dried at 40°C. Staining levels (*K/S*) were measured at 650 nm. Protein contents on the fabric were measured by the modified Lowry method [5].

The washing experiments testing the type of cellulose binding domain (CBD) involved 2.49 g mercerized and pre-washed (20 minutes, 50°C, 40 rpm in a Rotowash) cotton fabric washed for 20 minutes in 49 ml of cellulase solutions (pH 7, 0.1M $\text{KH}_2\text{PO}_4/\text{NaOH}$) of different concentrations. Cellulases were EGV and EGV-core (without CBD) of the fungus *Humicola insolens* delivered by Dr. M. Schüle in of Novo Nordisk, Denmark, and CenA (total cellulase) and CenA-core (CenA without CBD) of the bacteria *Cellulomonas fimi* (for preparation see reference 1), supplied by Professor D. Kilburn from the Department of Microbiology and Immunology of the University of British Columbia, Canada.

Protein in solution was determined before and after incubation by the Bradford method [2]. After incubation, a 1 ml sample was taken, 0.124 g of indigo (50 mg/g) was added, and fabrics were incubated another 20 minutes at 50°C (Rotowash, 40 rpm). Afterward, each fabric was rinsed with 400 ml water, washed for 20 minutes with 100 ml of distilled water at 50°C (Rotowash, 20 rpm), and oven-dried (60°C). Staining levels (*K/S*) were measured at 660 nm with an ACS II Spectrometer. Since fabrics were quite unevenly stained, *K/S* values represent the average of fifteen reflectance measurements on each sample. Average values coincided well with a visual comparison of the fabrics.

A previous indigo stained fabric had been prepared by adsorption with a total crude mixture of *Trichoderma reesei* cellulases (TC) and further washing in 10 L of ice-cold buffer (pH 4.85, acetic acid/NaOH) in a domestic washing machine. The control fabric was just washed with buffer. After 20 minutes, a fabric sample was taken and 46.4 mg indigo/g fabric was added. Washing continued for a further 20 minutes. To remove excess indigo, fabrics were washed for 20 minutes in cold water (washing machine), rinsed, and line dried. Protein contents on the fabric measured by the modified Lowry method [5] were 0 and 3.99 mg/g.

The experiments to test the after-washing step involved 2 g of these indigo stained fabrics pre-adsorbed with or without cellulases. The fabrics were washed at different levels of mechanical action (low level of mechanical action—LLMA = 20 rpm, high level of mechanical action—HLMA = 40 rpm + 5 steel discs) in 50 ml of a washing solution (liquor ratio = 1:25, Rotowash, 30 minutes, 50°C). Afterward, samples were rinsed and washed with 100 ml of distilled water (shaker at 100 rpm, 50°C, 20 minutes) and dried at 50–60°C. *K/S* values were measured at 650 nm before and after washing. Color removal was calculated in % of initial *K/S*. The different washing solutions were distilled water, 2 g/L BS1006 (standard detergent), Aquaderm (alkaline protease, Novo Nordisk, 1.6 g/L in 0.02M buffer of pH 8.5), and Alkalase (alkaline protease, Novo Nordisk, 1.6 g/L in 0.02M buffer of pH 8.5). BS 1006 is a complex mixture of alkylbenzenesulfonates (8.0%), ethoxylated alcohols (2.9%), long chain sodium soaps (3.5%), tripolyphosphates (43.7%), sodium silicates (7.5%), magnesium silicate (1.9%), carboxymethylcellulose (1.2%), Na_2SO_4 (21.2%), and a salt of ethylene dinitril tetraacetic acid (0.2%).

Results and Discussion

The diagram in Figure 1 shows the relation between indigo dosage and staining levels. At concentrations above 300 g/g fabric, a beginning saturation was ob-

FIGURE 1. Staining levels (K/S) of a mercerized cotton fabric with indigo at pH 5 and 7.

served. At pH 5, staining levels, especially at indigo concentrations above 50 mg/g, were higher than at pH 7. This is known, and we can speculate that at pH 7, the cotton fabric surface could be highly negatively charged (at least from some minor carboxylic groups formed during bleaching of the white yarn), and thus the negatively charged indigo particles in the dispersion would be repulsed, whereas under more acid conditions such as at pH 5, the fabric surface would be less charged.

The amounts of dispersed indigo used in our experiments are much higher than free dye during enzymatic washing of denim, but the results show clearly that higher concentrations of dispersed indigo cause more backstaining on cotton. This may partly explain the differences in backstaining between neutral and acid cellulases: As reported by Klahorst *et al.* [7], acid cellulases are more aggressive on denim and are able to release more indigo from denim fabrics. Thus, more backstaining has to be expected. Furthermore, acid cellulases have a higher affinity to indigo than neutral cellulases [3, 5].

Contrary to expectations, fabrics pre-adsorbed with different concentrations of total crude mixture cellulases of the *Trichoderma reesei* caused less backstaining than untreated fabrics did. Backstaining decreased with increasing amounts of cellulase that had previously been adsorbed by the fabric (Figure 2). In a previous work [5], we found an opposite result with an inactive mutant of cellulase CenA of *Cellulomonas fimi* adsorbed onto the fabric. This may be because the binding to cellulose is done with different cellulose binding domains. *Trichoderma reesei* CBDs belong to family I and the CenA CBD belong to family II. Linder *et al.* [8] reported that the adsorption of family I CBDs to cellulose is completely reversible, while Boraston *et al.* [1] stated that adsorption of family II CBDs to cellulose is irreversible. Experiments by Linder and Boraston were done under "test tube"

FIGURE 2. Staining levels (K/S at 650 nm) of indigo on fabrics previously adsorbed with a total crude mixture of *Trichoderma reesei* cellulases.

conditions, and we recently reported that even cellulases with cellulose binding domains of family II (*e.g.*, of *Cellulomonas fimi*) desorb to a certain extent under high levels of mechanical agitation and at alkaline pH [4]. Another reason might be that the previously tested cellulase was inactive [6]. Cellulase adsorption and activity are known to influence each other, since the substrate is degraded while the enzyme adsorbs [9].

When we compare indigo staining of fabrics in the presence and absence of cellulases (see Figure 2), it seems that cellulases facilitate the removal of indigo rather than increasing indigo backstaining. A possible explanation for this is that part of the indigo is not directly adsorbed onto cotton but is indirectly adsorbed by means of cellulases, and the hydrophobic cellulase-cotton interactions are disrupted more easily than indigo-cotton interactions. As recently reported [3], the presence of soluble cellulases decreases indigo particle size, increases indigo dispersion, and thus increases the relative solubility of indigo. Soluble indigo-cellulase complexes or aggregates are probably formed.

The results of Figure 3 confirm our previous explanation that the CBD of the cellulase adsorbed by the fabric is the main reason for the different results obtained in Figure 2 and in our previous results [5]. We made adsorption experiments with cellulases EGV (with a CBD from family I) from *Humicola insolens* and CenA (with a CBD from family II) from *Cellulomonas fimi*, and stained the fabrics in a second step with indigo. Additionally, we tested the effects of EGV-core and CenA-core, which are the truncated cellulases without a CBD of EGV and CenA, respectively.

Increasing concentrations of cellulase proteins of neutral cellulases decrease the indigo staining levels, with

FIGURE 3. Staining levels (K/S at 660 nm) of indigo on fabrics previously adsorbed with purified neutral cellulases with and without CBD. EGV-core and CenA-core are the truncated forms (without CBD) of EGV from *Humicola insolens* and of CenA from *Cellulomonas fimi*, respectively.

the exception of CenA, which remains unchanged (see Figure 3). The truncated cellulases without cellulose binding domains show lower degrees of indigo staining than the entire proteins EGV and CenA. These results can be explained by the missing CBDs and their special hydrophobic interactions with cellulose, which increase the affinity of cellulases to cellulose [4]. Different staining levels in experiments with *Trichoderma reesei* and are probably due to higher washing temperatures (50°C compared to 25°C), higher pH (see Figure 2 and 3), and different indigo affinities of the cellulases [3, 5]. Furthermore, staining levels are affected by the final washing procedures. Only results obtained under exactly the same final washing conditions may be compared.

Previous results showed that staining levels increased in the presence of an inactive mutant of CenA [5]. Compared to our results with the active CenA, this seems to indicate that cellulase activity facilitates its desorption from cotton. This is understandable, since an active cellulase may cut all glycosidic bonds around its adsorption place. Even if it does not desorb completely, the cellulase might go back into the bath still fixed to a small oligosaccharide [9]. Therefore, CenA has better adsorption properties onto cotton than other cellulases in this study, but better possibilities to be desorbed than their inactive mutant.

The diagram in Figure 4 shows the effects of mechanical agitation, protease treatment, and detergent during washing of fabric stained with indigo onto cellulases and onto the fabric itself. For all four washing solutions, increased mechanical action during washing results in better removal of indigo. Changing from a low level of mechanical action (LLMA = 20 rpm) to a high level of mechanical action (HLMA = 40 rpm + 5 steel discs), the

FIGURE 4. Influence of the mechanical action (LLMA = low level of mechanical action, HLMA = high level of mechanical action) on the reduction of backstaining (color removal in % K/S) during subsequent washing with proteases, detergent, and buffer.

removal of indigo expressed by color loss or reduction in % of K/S at 650 nm is at least twice the rate.

We achieved the best results with the standard detergent BS1006. For indigo stained fabric without previous adsorption of cellulase, the effects of the buffer and the alkaline proteases Aquaderm and Alcalase were appropriately the same, whereas the protease solutions removed more color from the indigo stained fabric with previous cellulase adsorption than the buffer solution. Even at LLMA, the reduction in K/S was 25–50% higher than with the buffer of pH 8.5. We suppose those differences in color loss for the protease and pH 8.5 buffer solution must be attributed to protease activity, which coincides with results reported by Clarkson *et al.* [6]. Our results, compared to previous ones from experiments at very low agitation in a Roach dyeing machine, indicate that the protease can act only on the cellulases at a higher degree of mechanical action. A possible explanation is that adsorbed indigo, which is not removed at low levels of mechanical action, protects the fabric bound cellulases against proteolytic attack. At higher agitation levels, indigo agglomerates probably diminish in size, so that cellulases become more exposed to protease action. Results show that proteases in washing solution contribute only by reducing the backstaining caused by indigo-cellulase-cellulose interactions, whereas indigo-cellulose interactions are not affected.

Conclusions

The results of this paper confirm that the reduction of indigo staining in the presence of cellulases is due to the "solubilization" of insoluble indigo by means of its af-

finity for the soluble cellulases. Part of the indigo set free by the cellulolytic activity of cellulases remains in the bath, aggregated with the cellulase enzymes, and less backstaining is the result.

Indigo backstaining decreases with increasing concentrations of cellulases with CBDS of family I (fungal), whereas backstaining is unchanged by cellulases with CBDS from family II (bacterial). Truncated cellulases without CBDS cause less backstaining than entire enzymes. These findings are interesting when tailoring new cellulases for enzymatic washing. If cellulases maintain sufficient cellulolytic activity without CBDS, backstaining may be reduced. Furthermore, as we saw, cellulases with CBDS of family I reduce backstaining, whereas cellulases with CBDS from family II do not.

For the removal of adsorbed indigo in the post-washing step, a high degree of mechanical action during washing is desirable, along with a good detergent. Proteases contribute to stain removal only under high mechanical action; otherwise we assume that adsorbed indigo protects cellulases from proteolytic attack. Washing experiments of indigo stained cotton fabrics also indicate that it is easier to attack hydrophobic cellulase-cotton interactions than interactions between indigo and cotton.

The indigo-cotton interactions seem to be the major cause of the remaining backstaining after post-washing steps, since most cellulases used in the denim washing are fungal (with a CBD of family I) and desorb completely during post-washing.

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Literature Cited

1. Boraston, A., Bray, M., Brun, E., Creagh, A. L., Gilkes, N. R., Guarna, M. M., Jervis, E., Johnson, P., Kormos, J., McIntosh, L., McLean, B. W., Sandercock, L. E., Tomme, P., Haynes, C. A., Warren, R. A. J., and Kilburn, D. G., The Structure and Function of Cellulose Binding Domains, in "Carbohydratases from *Trichoderma reesei* and Other Microorganisms," M. Claeysens, W. Nerinckx, and K. Piens, Eds., The Royal Society of Chemistry, Cambridge, 1998, pp. 139–146.
2. Bradford, M., A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding, *Anal. Biochem.* **72**, 248–255 (1976).
3. Campos, R., Andreas, J., Gübitz, G., and Cavaco-Paulo, A., Indigo-Cellulase Interactions, *Textile Res. J.* **70**, 532–536 (2000).
4. Cavaco-Paulo, A., Morgado, J., Andreas, J., and Kilburn, D., Interactions of Cotton with CBD Peptides, *Enz. Microbiol. Technol.* **25**, 639–643 (1999).
5. Cavaco-Paulo, A., Morgado, J., Almeida, L., and Kilburn, D., Indigo Backstaining During Cellulase Washing, *Textile Res. J.* **68**(6), 398–401 (1998).
6. Clarkson, K., Lad, P., Mullins, M., Simpsom, C., Weiss, G., and Jacobs, L., Enzymatic Compositions and Methods for Producing a Stone Washed Look on Indigo-Dyed Denim Fabric, World patent PCT WO1994-29426, 1994.
7. Klahorst, S., Kumar, A., and Mullins M., Optimizing the Use of Cellulase Enzymes, *Textile Chem. Color.* **26**(2), 13–18 (1994).
8. Linder, M., and Teeri, T., The Cellulose-Binding Domain of the Major Cellobiohydrolase of *Trichoderma reesei* Exhibits True Reversibility and High Exchange Rate on Crystalline Cellulose. *Proc. Nat. Acad. Sci.* **93**, 12251–12255 (1996).
9. Reinikainen, T., "The Cellulose-Binding Domain of Cellobiohydrolase I from *Trichoderma reesei*," vol. 206, VTT Publications, 1994.

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