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Indigo Backstaining During Cellulase Washing

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ABSTRACT

We have attempted to understand the mechanisms of indigo backstaining during enzyme stone washing by separately analyzing dye staining levels on different cotton surfaces during the process. The high ability of the cellulase enzyme protein to bind to cotton cellulose is the major cause of backstaining. Further studies of commercial cellulases show that the pH of maximum cellulase activity may not be the same pH of maximum of cellulase binding capacity to the substrate, and also that indigo dyes have completely different affinities for cellulase proteins from different fungal origins.

The production of "aged" denim garments with cellulases is the most successful enzyme process that has emerged in the textile industry in the last decade. The aged look is obtained by nonhomogeneous removal of the indigo dye trapped inside the fibers by the cooperative action of the enzymes and mechanical factors such as beating and friction. During treatment, the removed indigo can backstain the reverse side of the fabric if acid cellulases (optimum at pH 5) are used, while neutral cellulases (optimum at pH 7) yield very low dye redeposition [12]. This phenomenon remains unclear and cannot be explained without a basic understanding of the cellulase ageing process.

Cellulase enzymes will cleave to cellulose chains, producing a reducing end and a nonreducing end for each broken 1-4 ß-D glycosidic bond. The cleavages are made in a synergistic fashion by three classes of enzymes: endoglucanases (EG) that randomly hydrolyze cellulose, cellobiohydrolases (CBH) that split cellobiose from the chain ends, and cellobiase that splits cellobiose to glucose. The cooperative effect of mechanical action with cellulase activities (especially with EG activity) will produce soluble (chains of less than six residues are soluble) and insoluble sugars [2–4] along with the indigo dye trapped inside the fiber [8]. The enzymatic hydrolysis of insoluble cellulose is preceded by a strong adsorption step in the substrate [4] in such a way that for most cellulases, desorption happens only partially [1, 10]. Therefore, the liquor is expected to contain reducing soluble and insoluble sugars, nonadsorbed cellulase proteins, insoluble indigo dye, and some other additives like buffers, wetting agents, and dispersants. Also, from what we mentioned earlier, the undyed cotton fiber surface during the ageing process could be in one of the three forms: an intact cellulose surface untouched by the enzyme, a cellulose surface with reducing ends produced by enzymatic hydrolysis after enzyme desorption, and a cellulose surface where the enzyme protein is adsorbed. Backstaining is therefore likely to depend on the species present at the cotton fiber surface where dye redeposition is verified.

In this paper, we investigate the effect of several factors potentially related to indigo backstaining, like the different species present on the undyed cotton fiber surface, the pH of the liquor, and the kind of cellulase enzyme used.

Experimental

The original bleached cotton fabric we used represents an intact cellulose surface. A cellulose surface with a high concentration of reducing end groups was prepared by treating with 1.0 *M* HCl for 15 minutes at 80°C. A cellulose surface adsorbed with enzyme was simulated by a fabric adsorbed with CenAD392A protein, which is a catalytically inactive mutant of Endoglucanase A from *C. fimi* [6], *i.e.*, a cellulase with no enzyme activity remaining but a high binding affinity to cellulose. The bleached fabric was incubated with a

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cell extract of CenAD392A at pH 7 (0.05 *M* phosphate buffer) for 20 minutes at 35°C. The protein-treated fabrics were rinsed several times with warm water (50°C) and cold water to remove the unbound material and then line dried. This process was repeated three times, always reusing the cell extract of the previous step, obtaining four fabrics with different concentrations of bound protein.

The staining experiments were done on 1.5 g of fabric with 50 mg of indigo dye (BASF) and 50 ml of liquor in a Linitest machine for 30 minutes. The liquor was distilled water or buffer solutions (Titrisol buffers from Nordisk Merck) as indicated in the figures.

Commercial enzymes Denimax Acid P (acid cellulase) and Denimax T (neutral cellulase) from Novo were used at 50 EGU units [7]. The experiments were done with two fabric (original bleached cotton) pieces, 1.5 g and 0.6 g, with buffer and enzyme for 20 minutes at 40°C. The shorter pieces were taken out of the Linitest pot for bound protein determination after drying. Indigo dye (5 mg) was added to the remaining liquor containing the larger fabric piece and incubated for a further 30 minutes.

Indigo staining levels were measured as *K/S* values at 600 nm in a reflectance spectroscope (ACS Spectra Color) as a average of ten measurements on the same sample (errors bars indicated in the figures). Protein bound to the fabric was measured by the Lowry method [9] using a 50 mg of piece of fabric, based on the fact that the first Lowry alkaline reagent will desadsorb all bound protein into solution. Bovine serum albumin (Merck, pa) protein was used as the standard (errors of protein measurements were less than 2%).

Results and Discussion

The parameters of the prepared cotton samples are shown in Table I, and their indigo staining levels are shown in Figure 1. The cellulase treatment produced far fewer reducing ends than the acid hydrolysis (Table I), so if the presence of the reducing ends increases indigo staining levels, this can be verified on the acid-treated sample. This was not the case, since very little difference could be verified between indigo staining levels of the original and acid-treated samples (Figure 1). The formation of reducing ends at the fabric surface during the enzymatic ageing process seems to have little effect on indigo backstaining, since the reduction of indigo at pH 5-7 is not very effective and is also due to the lower affinity already reported [8] of the non-ionic leuco or reduced form of indigo for cellulose essentially present at neutral and acidic pH levels. At pH 9, the level of deposition on the acid-treated sample increased 1.5 fold relative to the untreated control.

TABLE I. Parameters of the cotton fabric samples.

	Bound protein, mg/g cotton	Reducing power[a], mg glucose/g cotton
Original	0	0.2
Cellulase treated[b]	–	0.9
Acid treated	0	1.5
CenAD392A protein adsorbed	1.5	0.2

[a] Measured as previously described [3]. [b] Reference values measured up to 10% of weight loss [3, 4].

The hypothesis that the reducing ends produced during the enzymatic ageing process might induce indigo backstaining is a valid one, since the reduction of indigo dye by glucose at pH 5 does happen, even if it happens only to a very small extent. This can be checked by heating an equimolar mixture of glucose and the dye in a pH 5 buffer for 15 minutes at 50°C, yielding a yellow filtrate caused by the presence of the reduced leuco form.

The results of Figure 1 indicate that the presence of the enzyme protein adsorbed on the fabric increases staining levels three-fold relative to the original fabric. The observed fabric with bound protein yields high levels of backstaining, justifying a more extensive study of this phenomenon. Figure 2 shows a linear relationship between the level of staining and the amount of bound protein, with the exception of the control fabric, *i.e.*, the fabric with no bound protein. In this control, the *K/S* value is much lower than the extrapolated zero value of the linear relation of *K/S* versus bound protein. This suggests that the dispersed indigo dye binds only to the enzyme protein through the numerous ionic groups present, and that the interactions between the

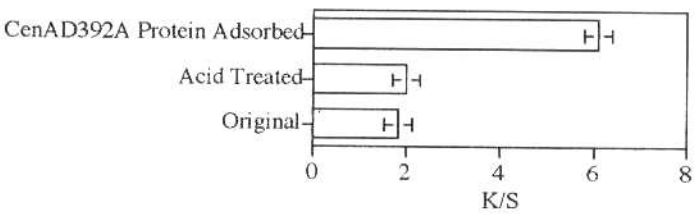

FIGURE 1. Staining levels (*K/S*) measured in distilled water for different fabrics.

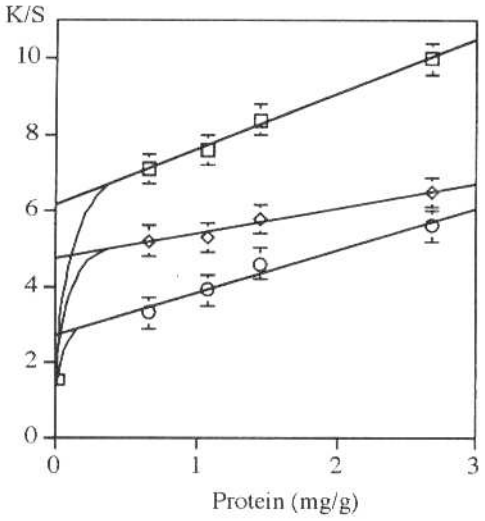

FIGURE 2. Staining levels (K/S) measured in different buffers versus concentration of the protein bound to the fabric: □ pH 9, ◇ pH 7, ○ pH 5.

dispersed indigo and cotton cellulose are incomparably lower when compared to the interactions between the dye and the protein.

The levels of backstaining appear to increase with pH (Figure 2), but a more detailed analysis of Figure 2 indicates that the slope between staining levels and protein concentration is lower at pH 7 and higher at pH 9, showing the importance of pH to the affinity between protein and indigo dye. Apparently, indigo dye has a lower affinity for *C. fimi* protein at neutral pH levels.

Figures 3, 4, and 5 display the results for commercial cellulases normally used for ageing denim fabrics. The relation of levels of staining versus pH (Fig. 3) is similar to the relation of bound protein versus pH (Figure 4), confirming that the ability of enzymes to bind on the cotton substrate plays a major role in indigo backstaining. The binding behavior of the acid cellulase (from *Trichoderma reesei*) is greatest at pH 5, the pH of maximum cellulase activity, while for the neutral cellulase (from *Humicola insolens*), the lower binding behavior at pH 7 is the pH of maximum cellulase activity (Figure 4). This unusual behavior of *Humicola* cellulases might be due to different pH maxima shown by their CBH enzymes relative to the all-crude mixture [14], but the pH binding profile on cotton of the individual components needs to be studied further.

The trials with commercial cellulases were based on the same activity (50 EGU units), but this means a

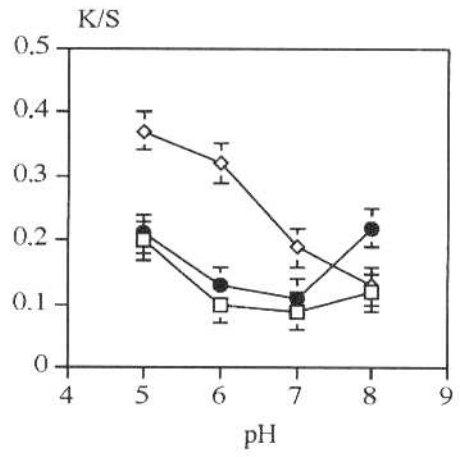

FIGURE 3. Relation between staining levels (K/S) and pH: □ no enzyme, ◇ acid cellulase, ● neutral cellulase.

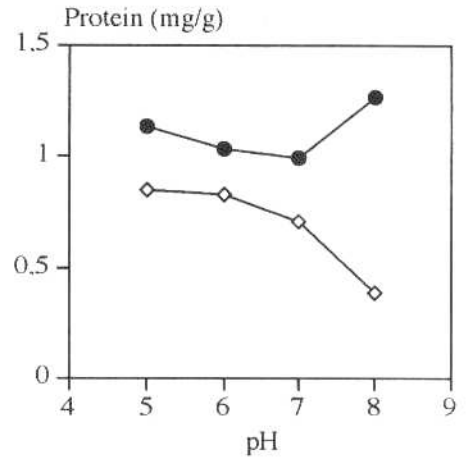

FIGURE 4. Relation between protein bound to the fabric and pH: ◇ acid cellulase, ● neutral cellulase.

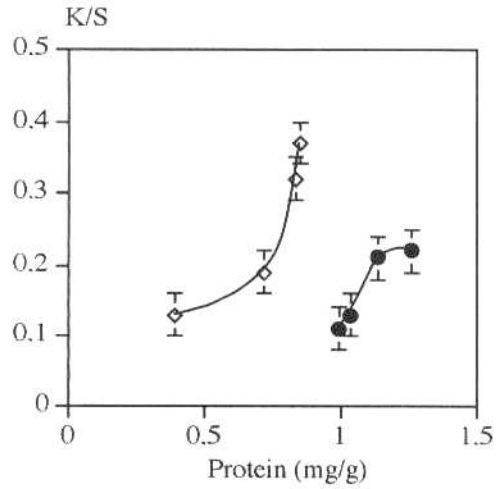

FIGURE 5. Relation between staining levels (K/S) and protein bound to the fabric: ◇ acid cellulase, ● neutral cellulase.

higher amount of protein was used for the neutral cellulase (Figure 4), because acid cellulase is much more aggressive than the neutral [11]. Neutral cellulase, despite its high concentration, gives lower staining levels when compared with the acid (Figure 5). These results also indicate that the indigo staining depends on the nature of the enzyme protein, *i.e.*, indigo dye affinity will depend on the external ionic residues of the globular enzyme protein, which seems to be different for *Humicola* and *Trichoderma* cellulases.

Conclusions

The results of this work confirm Clarkson *et al.*'s previous hypothesis that dye binding to the bound enzyme protein is the main reason for backstaining [5]. This group patented a process in which they suggested the use of proteases for reducing backstaining. The binding ability of an enzyme to a substrate is due to the presence of a substrate-binding domain [10], and this group further suggested the elimination of this binding domain to achieve low indigo staining levels. In our view, this seems counter productive, because eliminating the binding domain will greatly reduce the activity of the enzymes towards a high crystalline substrate like cotton [10]. Possibly there is no backstaining because there are no ageing effects.

The fact that indigo backstaining is reduced by several surfactants and desorption of cellulase from cellulosic fibers is enhanced by a wide range of different surfactants [13] also suggests that indigo comes into the liquor bound to enzyme proteins.

The earlier way to look for new cellulase systems was to search for enzymes active at higher pHs to prevent backstaining but still active enough to yield a good colour contrast. Our results suggest, however, that the major issue in preventing backstaining is not the pH but an enzyme that has very little affinity for indigo dye.

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