Indigo-Cellulase Interactions

RUI CAMPOS AND ARTUR CAVACO-PAULO[1]

Departamento de Engenharia Têxtil, Universidade do Minho, P-4800 Guimarães, Portugal

JÜRGEN ANDREAUS

Têxtil Alberto de Sousa, S.A., V. N. de Sande, 4802-Guimarães, Portugal

GEORG GÜBITZ

Institut für Mikrobiologie und Abfalltechnologie, Technische Universität Graz, A-8010 Graz, Austria

ABSTRACT

We have studied the affinity of cellulases from different fungal origins for insoluble indigo dye. Adsorption studies have shown that "acid cellulases" from *Trichoderma reesei* have a higher affinity for indigo dye than "neutral cellulases" of *Humicola insolens*. The particle size of indigo dye agglomerates is influenced by cellulase origin and concentration. Evidence shows that the nonpolar residues present in higher percentages in the neutral cellulases of *H. insolens* seem to play an important role in the agglomeration of indigo dye particles and probably in the reduction of backstaining. Furthermore, we investigate the effects of different levels of mechanical action on cellulase adsorption on undyed and indigo dyed fabrics.

The aged look of blue jeans can be obtained by nonhomogeneous removal of indigo dye trapped inside the cellulose fibers by the cooperative action of enzymes and mechanical action. However, redeposition of removed indigo dye on the reverse side of denim fabrics during washing with cellulases turns out to be a major problem. The first reports about backstaining were empirical observations that acid cellulases (optimum at pH 5) showed higher redeposition than neutral cellulases (optimum at pH 7) [4]. Using proteases during cellulase washing was believed to reduce cellulase binding to cellulose and therefore indigo redeposition on the cellulase proteins [5].

The nature of indigo-cellulase-cellulose interactions was first investigated in detail by Cavaco-Paulo *et al.* [4], who determined dye staining levels on different cotton surfaces. They attributed the effect of backstaining to indigo-cellulase affinities and demonstrated that the strong ability of cellulase proteins to bind to cotton cellulose is the major cause of backstaining. Studies with commercial cellulases showed that indigo had different affinities for cellulase proteins from different fungal origins [4].

The lack of information in the literature and the high interest in solving problems related to backstaining justify the need for more detailed scientific and technical studies. Our investigations focus mainly on indigo-cellulase interactions in the absence of cotton cellulose. We also compare the absorption of acid *Trichoderma reesei* and neutral *Humicola insolens* cellulases on indigo dyed and undyed fabrics.

Experimental

For the indigo-cellulase interaction experiments, 25 mg insoluble indigo (BASF, ref. 797 6670) dye was incubated at 25°C in solutions (50 ml) of 0.0–1.0 g l^{-1} protein of crude cellulase preparations from *Trichoderma reesei* (supplied by Rohm Enzyme Finland, Ltd.) and *Humicola insolens* (supplied by Novo Nordisk, Ltd.) under mechanical agitation on an IKA-Vibrax-VRX at 200 rpm for 20 hours. Subsequently, the mixtures were vacuum-filtered and the adsorption of cellulase protein onto the indigo dye was determined, measuring protein in solution with the Bradford method [2]. The same indigo and cellulase amounts were mixed on a shaker (100 rpm) at 50°C for 20 hours, and indigo particle size was determined with a Malvern Mastersize apparatus. The amino acid composition of the crude cellulase preparations was determined after acid hydrolysis and chromatographic analysis by standard methods [1].

[1]To whom correspondence should be addressed.

For each enzymatic adsorption treatment, we used undyed and indigo dyed (BASF 4B-D150 %, ref. 797 7058) fabrics (0.86 g g^{-1}). The cold exhaustion process (BASF Technical Catalogs, IK CIII) was used to dye the fabrics. For the treatment with crude cellulase preparations, fabric samples (1 g) were placed in steel containers (400 ml) of a Rotowash/Autowash MKII series 7227 laboratory machine. A buffered solution at pH 5.0 (50 ml, 0.06 *M* acetate buffer) with 5 mg g^{-1} protein/fabric was added and incubated at 50°C for 60 minutes at 20 and 40 rpm. Subsequently, fabrics were rinsed and dried and their adsorption of cellulase protein was determinated using the Bradford method [2]. For the *H. insolens* cellulase adsorption treatments, the procedure was identical, except that the pH was adjusted to 7.0 (0.06 *M* phosphate buffer).

We studied the interaction of indigo and isopeptides using poly-L-serine and poly-L-valine (Sigma): 25 mg isopeptide and 25 mg insoluble indigo dye were incubated in an aqueous solution (50 ml) in an Ahiba Spectradye machine at 40°C and 60 rpm for 16 hours. Subsequently, we determined indigo particle size with a Malvern Mastersizer.

Results and Discussion

Indigo-Cellulase Adsorption

Interactions between indigo and cellulases (Figure 1) seem to be comparable to common physicochemical adsorption phenomena, which may be described by the Langmuir model [3]. The same model was used previously for adsorption of cellulases on cotton [3]. We calculated estimated adsorption constants (K_a) and estimated saturation levels of *Trichoderma reesei* and *Humicola insolens* cellulases on indigo dye based on the Langmuir model by nonlinear regression analysis (Table I). The isotherms are shown in Figure 1.

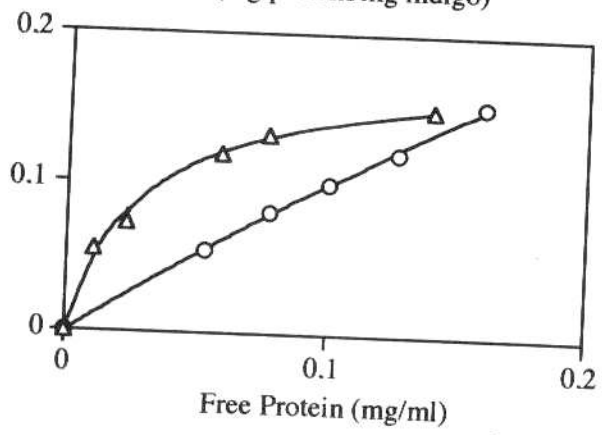

FIGURE 1. Langmuir isotherms of protein on insoluble indigo dye: △ *Trichoderma reesei*, ○ *Humicola insolens*.

TABLE I. Parameters of protein adsorption in insoluble indigo dye.

Cellulase	K_a, ml/mg	Saturation level, mg mg^{-1} indigo/protein
Trichoderma reesei	35 ± 5	5.6 ± 0.3
Humicola insolens	1.0 ± 0.2	0.9 ± 0.2

The isotherm for *T. reesei* cellulases shows a typical saturation profile, whereas the isotherm for *H. insolens* cellulases increases almost linearly with free enzyme concentration. The calculated parameters and the respective Langmuir isotherms indicate that indigo has a higher affinity for acid cellulases (*T. reesei*) than for neutral cellulases (*H. insolens*). This can be seen by the higher K_a value and higher saturation levels on the acid cellulases, which can be explained by an increased number of adsorption sites for indigo on the acid cellulase molecules. These results coincide with the findings of Cavaco-Paulo *et al.* [4], who previously showed that acid cellulases adsorbed onto cotton fabrics cause higher indigo staining levels than comparable amounts of adsorbed neutral cellulases.

Acid and neutral cellulases have different amino acid compositions (primary structure), and thus they differ in their secondary and ternary structures. Amino acid compositions of both cellulases are shown in Figure 2. Differences in amino acid residues of acid and neutral cellulases seem to be the main reason for their different binding behaviors to indigo dye. This means that indigo backstaining depends on the nature of the protein. Indigo dye affinity might depend on surface ionic residues of the globular protein, which seems to be different for *T. reesei* and *H. insolens* cellulases [4].

Indigo Particle Size

Insoluble indigo is known to form agglomerates in aqueous solutions. As we found by particle size measurements (Figure 3), indigo particle size can be influenced by cellulase enzymes. We found completely different behaviors for *Trichoderma reesei* and *Humicola insolens* cellulases. For acid cellulases, the size of the indigo dye particles decreased with the addition of low concentrations of acid cellulase protein and remained unchanged for increasing protein concentrations above 0.2 mg ml^{-1}. This may be because the saturation equilibrium of acid cellulases with indigo was reached at about 0.32 mg ml^{-1}. In contrast, low concentrations of neutral cellulases led to an increase in indigo particle size with a maximum of around 0.2 mg ml^{-1} protein. However,

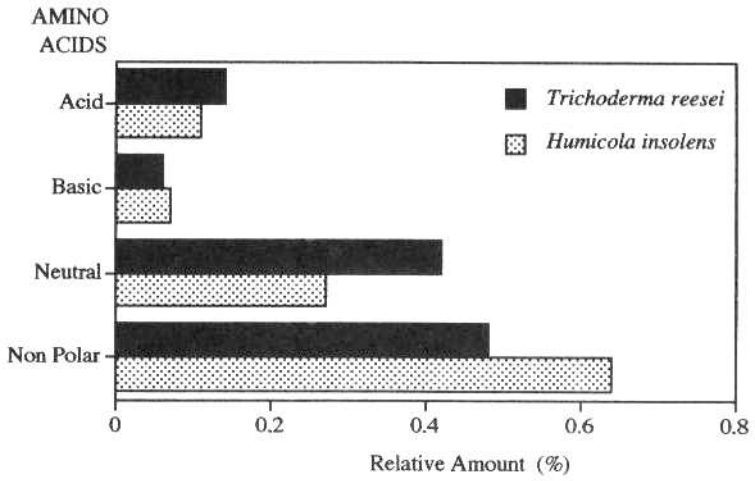

FIGURE 2. Relative contents of aminoacid composition of *T. reesei* and *H. insolens* cellulases.

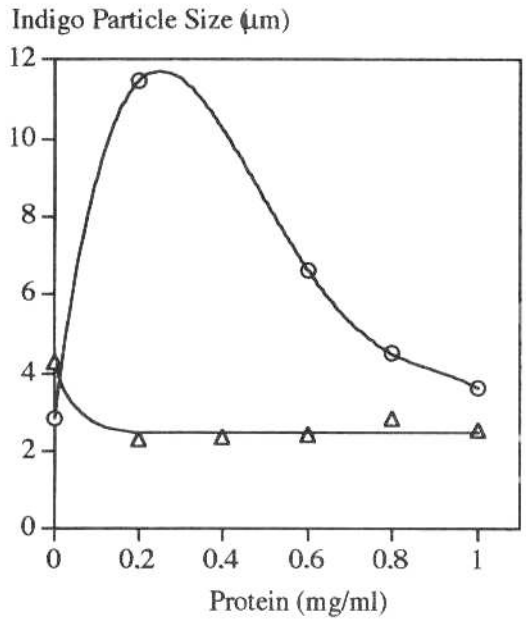

FIGURE 3. Indigo particle sizes at different enzyme concentrations: △ *T. reesei*, ○ *H. insolens*.

aggregation decreased with higher protein concentrations. At about 0.2 mg ml^{-1}, indigo agglomerates incubated with neutral cellulases were six times bigger than those with acid cellulases.

In aqueous solution, indigo particles tend to be in dispersion. When soluble neutral cellulase protein is added to this dispersion, indigo particles form agglomerates. This phenomenon could be explained by a competition between indigo and neutral cellulases for solvation water. Indeed, neutral cellulases are soluble, but have less affinity for indigo and use more water for solvation. By this process, indigo particles agglomerate. In contrast, indigo agglomerates are fractionated into smaller particles when incubated with acid cellulases from *T. reesei*. These acid cellulases have a higher affinity for indigo than neutral cellulases [4]. Thus, binding of indigo molecules to acid cellulase may somehow separate agglomerates. When cellulases are preadsorbed onto cellulose, indigo binds to them and increases backstaining.

INDIGO-ISOPEPTIDE INTERACTIONS

We tried to identify the amino acid residues responsible for the different binding behaviors of acid and neutral cellulases to indigo. We found big differences in the contents of nonpolar and neutral amino acids for both cellulase types (Figure 2). Comparing single amino acid contents, we selected two different isopeptides for experiments to elucidate interactions between amino acids and indigo. We took isopeptides, polymers of identical amino acids, as representatives of single amino acid residues on the protein surface. The interactions occurring between proteins (cellulases) and indigo were simulated by incubating isopeptides with indigo in water and measuring the particle size of the resulting dispersion. We assumed the particle size of indigo agglomerates provided some information about indigo-amino acid interactions.

Figure 4 shows the diameter distribution of particles formed between indigo and isopeptides in aqueous solution. Distributions had two maxima: compared to the distribution of indigo particle size, the first maximum, at a low particle diameter of the indigo-isopeptide agglomerates, seemed to be shifted to higher diameters, whereas the second maximum, at a higher particle size, seemed to

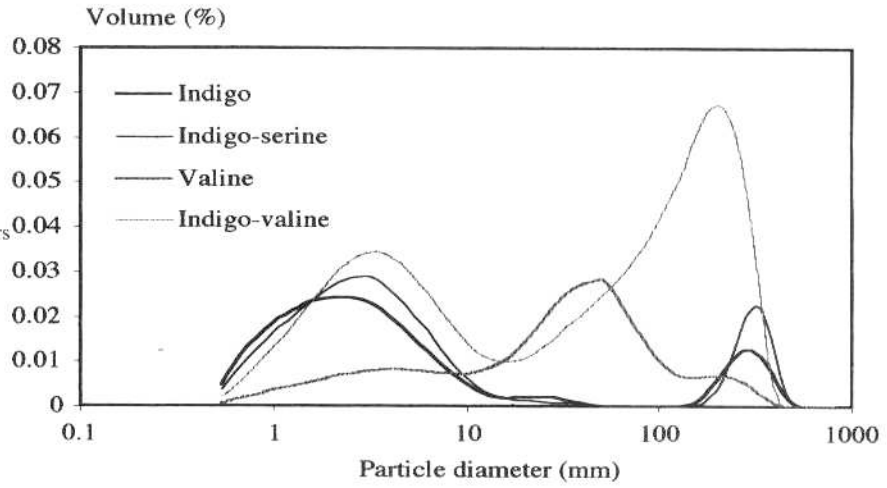

FIGURE 4. Distribution of particle diameters formed between indigo and isopeptides.

be shifted to smaller diameters. When indigo was incubated with valine, the second maximum was much higher than the first maximum at smaller particle size and the second maximum obtained with the other isopeptide. This may indicate that valine residues tend to agglomerate indigo particles.

We measured particle size with a Malvern Mastersizer, which calculates the particle mean diameter either from the particle volume ($D[4, 3]$) or from the surface area ($D[3, 2]$). We obtained different results for these two diameter measurements with our experiments for all incubation mixtures, which is a strong indication that particles are far from being spherical. $D[4, 3]$ is the more efficient way to define particle diameter. A comparison of $D[4, 3]$ shows that serine (completely soluble) and valine ($D[4, 3] = 51$ μm) tend to increase indigo agglomerates from 43.5 μm to 56.8 and 88.9 μm respectively.

Serine is a neutral residue and is present in a higher relative amount in cellulases of *T. reesei* than in *H. insolens* cellulases. Since serine is completely water soluble, a competition for water may occur between indigo and the residue molecules, thus facilitating indigo aggregation.

Valine as a nonpolar residue is present in a higher relative amount in *H. insolens* cellulases than in *T. reesei* cellulases. It is insoluble in water and shows the worst results relative to indigo dispersion. We can't draw general conclusions about the behavior of protein-indigo interactions based on just two amino acid residues, but serine and valine are good representatives of neutral and nonpolar residues, and our results seem to agree with previous results.

LEVELS OF PROTEIN ADSORPTION

As Figure 5 shows, cellulase adsorption is enhanced by mechanical action. This may be due to the increased availability of adsorption sites at high levels of mechanical action, as previously reported [3]. At a higher level of mechanical action, *H. insolens* cellulases show a higher degree of adsorption on undyed fabrics, whereas *T. reesei* cellulases adsorb more to indigo-dyed fabrics. At low levels of mechanical action, both cellulases adsorb more to indigo-dyed fabrics. *Trichoderma* cellulases seem to have more affinity for indigo-dyed cellulose. We can conclude that at high levels of mechanical action, *H. insolens* has a lower affinity for indigo-dyed cellulose and that cellulases are more easily desorbed (Figure 5), therefore inducing less backstaining.

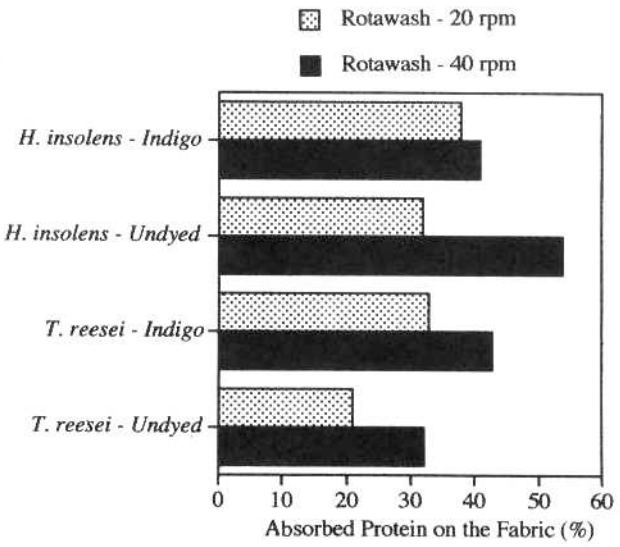

FIGURE 5. Levels of protein absorption in dyed and undyed cotton fabrics.

Conclusions

Our findings confirm previous results indicating that indigo has a higher affinity for acid cellulases (*Trichoderma reesei*) than for neutral cellulases (*Humicola insolens*) [4]. Cellulases from *T. reesei* with a higher content of neutral aminoacids show more affinity for indigo.

The results for particle size measurements of indigo agglomerates show completely different effects for *T. reesei* and for *H. insolens*. For neutral cellulases, indigo particle size decreases up to 12 μm with increasing protein concentration, and remains unchanged after reaching the adsorption maximum for protein (0.32 mg ml^{-1}). Particle size measurements of indigo-isopeptide complexes indicate that some amino acid residues may promote agglomeration. Considering the variability of measurements based on the volume or the surface area, however, other techniques such as adding surfactant should also be evaluated to study indigo-protein interactions.

Our results for protein adsorption on indigo dyed fabrics show that *T. reesei* cellulases are preferable for adsorbtion on to indigo-dyed fabrics.

Literature Cited

1. Bailey, P., "An Introduction to Peptide Chemistry," J. Wiley & Sons, NY, 1990.
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,** 248–254 (1976).
3. Cavaco-Paulo, A., Almeida, L., and Bishop, D., Hydrolysis of Cotton Cellulose by Engineered Cellulases from *Trichoderma reesei*, *Textile Res. J.* **68,** 273–280 (1998).
4. Cavaco-Paulo, A., Morgado, J., Almeida, L., and Kilburn, D., Indigo Backstaining During Cellulase Washing, *Textile Res. J.* **68,** 398–401 (1998).
5. Clarkson, K., Lad, P., Mullins, M., Simpsom, C., Weiss, G., and Jacobs, L., Enzymatic Compositions and Methods for Producing Stone Washed Look on Indigo-Dyed Denim Fabric, Patent PCT WO1994-29426.

Manuscript received January 19, 1999; accepted April 23, 1999.