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Hydrolysis of Cotton Cellulose by Engineered Cellulases from *Trichoderma reesei*

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ABSTRACT

We have characterized the activities of TC, EG-rich, and CBH-rich cellulases from *T. reesei* and have shown that their activities towards cotton fabrics are influenced by ionic strength and adsorbed ionic species as well as by temperature and pH. Adsorption and kinetic experiments confirm that increasing mechanical agitation favors EG attack by greatly increasing the availability of sites for EG adsorption. It is not clear whether this is a consequence of fiber fibrillation damage or of improved access to fiber surfaces deep within the fabric structure. The enhanced rate of cellulolytic hydrolysis of mercerized cotton and the inhibitory effects on reactive and direct dyed fabrics are explained mainly in terms of increased or reduced availability of adsorption sites for CBHs and EGs. The implications for textile finishing are far-reaching. It is clear that a fabric's processing history (especially mercerizing and dyeing), construction, and level of applied mechanical agitation can be as important as the choice of enzyme composition and concentration in determining the consistency and quality of the end result.

The catalytic activity of cellulase enzymes depends on their ability to adsorb on a substrate in such a way that the reaction site fits precisely into the reactive "tunnel" or "cleft" in the enzyme protein structure. The frequency with which this is successfully achieved (and which may be measured as the enzyme activity under specific conditions) therefore strongly depends on factors that affect the accessibility of the substrate and the conformation of the enzyme protein, as well as the relative concentrations of substrate and enzyme.

The physical form of cellulose clearly affects its accessibility, and the activities of cellulolytic enzymes, when measured against soluble cellulose derivatives, or short chain cellulose oligomers are therefore quite different from those measured against insoluble cellulose substrates. Similarly, the degree of swelling, orientation, and crystallinity of different cellulosics may be expected to lead to different rates of cellulolytic degradation [8, 9]. Furthermore, when the cellulose substrate is in the form of cotton or another cellulosic fiber fabric, the rate of cellulolytic attack is likely to depend on the hairiness of the fabric surface [13] and the relative tightness or openness of the yarn twist and fabric construction. Fabric processing history also affects the rate of cellulolytic attack. For example, whereas mercerizing increases the accessibility of cotton to cellulase [1, 8], reactive dyeing and direct dyeing both reduce the rate of hydrolysis by cellulase [10, 16].

In recent years, there has been considerable progress in elucidating the mechanisms of cellulolytic degradation [6, 20], and better understanding is improving and extending the scope of textile finishing processes that use cellulase [12, 13]. As we have noted before [13], the cellulase varieties used in textile finishing products are secreted by various microfungi as complex mixtures of endoglucanases (EG, EC 3.2.1.4), cellobiohydrolases (CBH, EC 3.2.1.91), and cellobiases (EC 3.2.1.21). Since EGs cause random scission of cellulose chains (producing new reducing and nonreducing end groups) and CBHs split cellobiose from the ends of cellulose molecules, the EGs and CBHs act synergistically to degrade cellulose. Cellobiase hydrolyzes cellobiose to glucose. The precise roles of the several different EG and CBH components that are normally present in total cellulase complexes (TCs) are, however, not yet fully understood in the context of crystalline cellulose degradation.

DNA techniques have made it possible to manipulate the genes of cellulase-secreting fungi so that derivative or "genetically engineered" fungal strains can be produced. In turn, these strains secrete new cellulase compositions in which one or more of the components are

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enriched or deleted [17, 22]. Studies with such engineered cellulase mixtures are helping to improve the understanding of cellulase finishing effects on textiles, and are leading to the marketing of more specific cellulase compositions that deliver desired benefits more cost effectively, such as stonewashed denims and depilled and low-pill finished cotton fabrics [13].

Despite these advances, we believe that much remains to be learned about the effective control of cellulase finishing processes. In addition to the variability introduced by different fabric constructions and processing histories, process variables such as the concentration or ionic strength of buffer solutions may be expected to affect the conformation of enzyme proteins and their interactions with the substrate. Note also that the level of mechanical agitation applied to fabric finishing processes appears to affect the relative rates of EG and CBH attack, measured as weight loss [13]. In earlier work [12], we reported that the "exo" activity of CBHs in a TC mixture was reduced by increasing mechanical agitation, whereas the "endo" activity of EGs increased. Thus, the choice of processing method may be expected to be a source of variability in the delivery of desired fabric finishing effects.

In this work, we further clarify some of these effects by characterizing the activities of a TC, an EG-rich mixture, and a CBH-rich mixture and measuring their activities towards different forms of cellulose. We also investigate the effects of ionic strength on their activities towards cotton fabric. We then measured the effects of mechanical agitation level on adsorption and initial hydrolysis rates of cotton fabric for the same three cellulase mixtures. In addition, we investigate the effects of mercerization and dyeing with vat, direct, and reactive dyes on the subsequent adsorption and reaction kinetics for the same TC, EG-rich, and CBH-rich mixtures.

Experimental

Cellulase varieties were supplied by Primalco Biotech (Finland). The TC was a cellulolytic complex secreted by the filamentous fungus *Trichoderma reesei*, which is known to contain a cellobiase, EG I, EG II, CBH I, and CBH II. This cellulolytic complex was believed also to contain EG III and EG V [21, 23]. Engineered strains of *Trichoderma reesei* were used to produce cellulase complexes in which the CBH I and CBH II were absent (TC-CBH I & II) and EG I and EG II were absent (TC-EG I & II). The three cellulase complexes were supplied as solution compositions under the codes CE 883042, CE 519/92, and CE 523/92 for the TC, TC-EG I & II and TC-CBH I & II, respectively. The activities of these complexes were characterized using methods described below. All treatments used a commercially scoured and bleached, plain woven, 100% cotton poplin with 60/32 ends/picks per cm, 0.5 mm thickness at 2.5 gf/cm^2, and 100 g/m^2 weight.

Enzyme Activity

We determined the activity of cellulase mixtures towards insoluble and soluble substrates at 50°C and pH 4.8 (0.1 *M* acetate buffer) on water soluble carboxymethyl-cellulose (CMC), BDH analar (high viscosity), and phosphoric acid swollen Avicel (PASA, prepared as described Evans *et al.* [14]) by means of the increased concentration of liberated soluble reducing sugars, using methods similar to those previously described by Evans *et al.* [14]. Activity towards cellobiose was determined using the glucose-oxidase assay method [14]. Activity on scoured cotton fabric was determined by measuring fabric weight loss after 6 days with no agitation, because agitation affects the rates of cotton hydrolysis differently for the three cellulase components [12]. Fabric weight loss was determined by weighing the fabric samples before and after treatment following 40 hours of conditioning at 20°C and 65% rh.

For the profiles of relative activity towards cotton, fabric samples (10 g) were treated at a liquor-to-fabric ratio of 10 ml/g in the stainless steel pots (500 ml) of a Linitest machine rotating at 65 rpm. All treatment solutions contained 5 ml enzyme preparation/liter, and their activity profiles were determined by means of soluble reducing sugars liberated after 1 hour at 50°C over the pH range 4.0 to 8.0 ("Titrisol" buffer solutions as received from Merck) and over the range 20–70°C at pH 5.0 (Titrisol buffer). The soluble reducing sugars were determined spectroscopically by formation of a colored cuprous neocuprione complex [12].

Relative activity of ionic strength effects, similar to those described above, was also determined at 50°C in acetate and citrate buffer solutions, all at pH 4.8 but of varying ionic strength (see Table II). Further activity was determined at pH 4.8 in 0.1 *M* acetate buffer to which had been added 10 g/l of either sodium chloride or calcium chloride (anhydrous).

Adsorption and Kinetic Experiments

Fabric samples (3.0 g) were treated at 50°C and pH 4.8 (0.1 *M* acetate) in solutions (100 ml) with concentrations ranging from 0.02 to 0.43 g total protein/liter for TC, TC-EG I & II, and TC-CBH I & II. These treatments were done in the stainless steel pots (250 ml) of the Linitest machine rotating at 65 rpm to provide a low level of mechanical action (LM). A higher level of me-

chanical action (HM) was provided in further experiments by adding seven stainless steel discs to the Linitest pots.

Adsorption of cellulase protein onto the fabric was determined by loss from solution after 30 minutes at LM and HM using the Bradford method [7]. Initial rates of reaction were measured, also after 30 minutes at LM and HM, by determining soluble reducing sugars [12].

There were similar experiments at LM only on mercerized samples and dyed samples. Mercerization was done industrially by ETA, SA - Guimarães. Dyeings used standard procedures to apply 3% Solophenyl Blue GL (C.I. Direct Blue 71), 3% Indanthren Red FBB (C.I. Vat Red 10), and 3% Remazol Brilliant Blue R (C.I. Reactive Blue 19).

Results and Discussion

All quoted results are mean values of three independent experiments. Protein and liberated reducing sugars were all determined in duplicate in each independent experiment.

Cellulase Activities on Soluble and Insoluble Substrates

The measured activities of TC, TC-EG I & II, and TC-CBH I & II towards CMC, PASA, and cotton fabric are given in Table I. The activities of TC-CBH I & II towards CMC and of TC-EG I & II towards PASA were each greater than those of TC. These results illustrate the expected increments in the classical EG and CBH activities of TC-CBH I & II and TC-EG I & II, respectively [15]. The activities of TC towards cellobiose, CMC, and PASA were all lower than those of TC-EG I & II, despite the fact that TC caused greater cotton weight loss. This apparent contradiction shows that care should be exercised in predicting cellulase activity on cotton from data on other forms of cellulose or its derivatives. The result also points to the importance of synergy between EGs, CBHs, and cellobiase in the hydrolysis of cotton cellulose.

TABLE I. Cellulase activities towards various cellulosic substrates at 50°C and pH 4.8 (0.1 *M* sodium acetate).

Substrate	TC	TC-CBH I & II	TC-EG I & II
Substrate[a]	2.2 U/g	4.4 U/g	4.7 U/g
CMC[b]	94 U/g	159 U/g	120 U/g
PASA[b]	194 U/g	93 U/g	275 U/g
Cotton[c]	63%	3%	52%

[a] One unit (U) liberates 2 μmol of glucose per minute. [b] One unit (U) liberates 1 μmol of reducing sugars (as glucose) per minute. [c] Weight loss after 6 days.

The deletion of CBH I and CBH II activity from the total crude mixture dramatically reduced cotton weight loss (Table I), thus confirming the importance of CBH activity in solubilization of the polymer. The known synergy between CBH and EG activities [18, 19, 24] leads to the expectation that deleting EG I and EG II should also greatly reduce cotton weight loss. The surprisingly high activity of TC-EG I & II was probably due to the remaining EG III and EG V activities in this crude mixture.

Activity Profiles and Ionic Strength Effects

The temperature and pH profiles of the three cellulase mixtures are shown in Figure 1, which reveals that 50°C and pH 5 are optimal conditions for all three mixtures. The optimum pH range for TC-CBH I & II is, however, broader and displaced to a slightly higher pH compared with those of the other two cellulase mixtures.

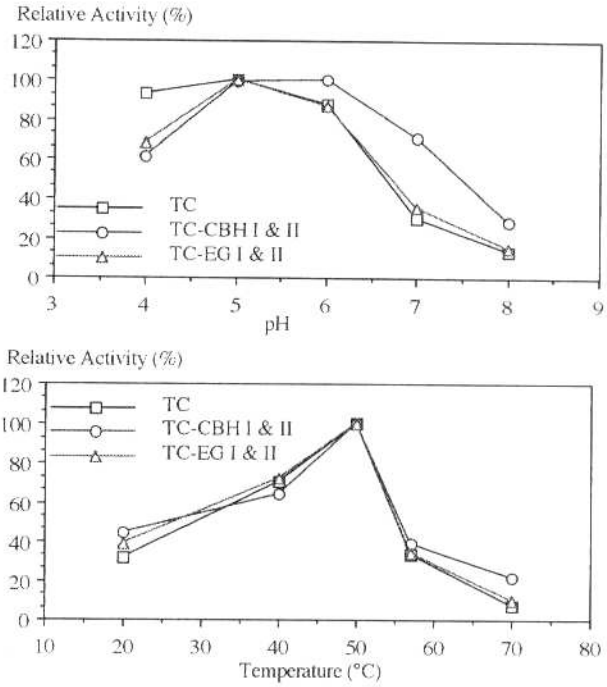

FIGURE 1. Temperature and pH profiles for the activity of *T. reesei* cellulase towards scoured and bleached cotton fabric relative to the performance of the individual mixtures at 50°C and pH 5 (which represents 100% in each case).

All three crude mixtures were similarly affected by changes in ionic strength and valency (Table II). Increasing electrolyte concentration compresses the electrical double layer and generally leads to increased adsorption of negatively charged species on

TABLE II. Effects of electrolyte concentration and ionic strength on cellulase activity (%) towards cotton fabric at 50°C and pH 4.8 (relative to 0.1 *M* acetate, which equals 100%).

Electrolyte	TC	TC-CBH I & II	TC-EG I & II
Citrate-1 *M*	25	18	23
Citrate-0.1 *M*	93	81	95
Acetate-1 *M*	78	95	74
Acetate-0.1 *M* (a)	100	100	100
NaCl-0,17 *M* in (a)	99	100	94
$CaCl_2$-0,09 *M* in (a)	70	70	62

cotton fibers. This might be expected to increase cellulase adsorption and so to increase hydrolytic activity. At pH 5, however, amino acid side chains in the cellulase proteins are protonated. Consequently, increased electrolyte concentration leads to increased adsorption of anions by the enzyme proteins, and hence to greater electrostatic repulsion between the cellulases and cellulose. This seems to be a possible explanation for the reduced cellulase activity towards cotton at the higher concentrations of the buffer solutions tested, and when acetate is replaced by citrate at the same molarity (see Table II). There may, however, be additional deactivating effects due to changes in conformation of the enzyme protein by adsorbed anions, particularly in the case of more bulky trivalent citrate ions. Note that the activity of the EG-rich complex (TC-CBH I & II) is affected relatively more by citrate ions and relatively less by acetate ions in comparison with TC and TC-EG I & II. This is not surprising since the protein structures of EGs and CBHs are very different [20] and may be expected to be affected differently by adsorbed ions.

The partial replacement of sodium by calcium ions might also be expected to reduce electrostatic repulsion between the negatively charged fiber and enzyme proteins, thus enhancing hydrolytic activity. The observed deactivating effect of divalent cations is, however, probably due to more specific effects of their adsorption on the cellulase proteins, where any calcium bridging may significantly change the stereochemistry of their cellulose binding domains or their reactive centers.

For our applications, it was clear that buffering with a low concentration of monovalent anions gave the highest cellulase activities, and all further experiments were buffered to pH 4.8 using the lowest practicable concentrations of acetate buffer. The implications for textile processing should not be overlooked: the choice of buffer and buffer concentration will each influence the cost effectiveness of cellulase finishing operations.

ADSORPTION AND REACTION KINETICS

Models Used

The hydrolysis of cellulose by cellulase is preceded by adsorption of the enzyme on the substrate. Since the rate of cellulase hydrolysis is relatively slow, we may assume that equilibrium adsorption of cellulase on the substrate is maintained throughout, provided that diffusion effects are avoided by maintaining a sufficiently high level of agitation. The adsorption/desorption process may be described by

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (ES)ad \quad ,$$

where E = enzyme, S = substrate, $(ES)ad$ = enzyme adsorbed on substrate, and k_1 and k_2 are the rate constants for adsorption and desorption, respectively. If the adsorption constant $K = k_1/k_2$, the Langmuir equation [2, 4, 5] gives

$$Eads = (EmK[Ef])/(1 + K[Ef]) \quad , \qquad (1)$$

where *Eads* is the amount of enzyme adsorbed at equilibrium (mg/g), *Em* is the amount of enzyme adsorbed at saturation (mg/g), and $[Ef]$ is the free enzyme concentration in solution at equilibrium (mg/ml). We used this model to calculate adsorption constants (K) and saturation levels (Em) on cotton cellulose for TC, TC-CBH I & II, and TC-EG I & II from the results of the adsorption experiments (see Tables III and IV). The procedure assumes that equilibrium adsorption of cellulase on cotton is achieved and maintained in our 30-minute experiments.

TABLE III. *Em* (mg/g) calculated from Equation 1 using nonlinear regression methods.

Fabric enzyme[a]	TC	TC-CBH I & II	TC-EG I & II
LM	16.7 ± 0.4	4.9 ± 0.4	7.8 ± 0.2
HM	24.5 ± 0.2	47.3 ± 9.9	10.1 ± 0.3
M.LM	26.1 ± 1.5	6.1 ± 0.2	10.6 ± 0.5
R.LM	13.6 ± 0.1	5.6 ± 0.2	1.1 ± 0.1
V.LM	16.5 ± 0.5	5.1 ± 0.2	7.4 ± 0.2
D.LM	7.2 ± 0.4	1.4 ± 0.1	8.4 ± 0.2

[a] LM and HM = cellulase treatments on scoured and bleached cotton at low and high levels of mechanical agitation, respectively. M.LM = cellulase treatments on mercerized, scoured, and bleached cotton at a low level of mechanical agitation. R.LM, V.LM, D.LM = cellulase treatments at a low level of mechanical agitation on scoured and bleached cotton dyed with reactive, vat, and direct dyes, respectively.

The Michaelis-Menten kinetic model [2] assumes that formation of an enzyme-substrate complex (ES) is a prerequisite for enzymatic reactions:

TABLE IV. K (ml/mg) calculated from Equation 1 using nonlinear regression methods.

Fabric[a]	TC	TC-CBH I & II	TC-EG I & II
LM	1.51 ± 0.04	6.54 ± 0.99	4.92 ± 0.18
HM	1.47 ± 0.02	0.37 ± 0.08	3.77 ± 0.20
M.LM	0.93 ± 0.06	6.56 ± 0.30	3.36 ± 0.24
R.LM	2.11 ± 0.01	4.33 ± 0.22	67.93 ± 27.07
V.LM	1.75 ± 0.07	7.68 ± 0.63	4.76 ± 0.26
D.LM	4.49 ± 0.37	9.38 ± 0.50	2.47 ± 0.09

[a] Symbols the same as for Table III.

$$E + S \underset{k_a'}{\overset{k_a}{\rightleftharpoons}} (ES) \xrightarrow{k_b} P + E \quad ,$$

where k_a and k_a' are the forward and reverse rate constants for complex formation, k_b is the reaction rate constant, and P is the reaction product. If the ratio of enzyme to substrate is always small and the substrate concentration may be varied within the limits of saturation by the enzyme, the reaction rate (V) is given by [2]

$$V = (Vm[S])/(Km + [S]) \quad , \qquad (2)$$

where Vm is the maximum rate of reaction (at enzyme saturation) and Km is the half saturation constant relative to the enzyme sites, *i.e.*, $Km = (k_a' + k_b)/k_a$.

Since it is usually preferable to express the rate of enzymatic reactions as a function of enzyme concentration (rather than substrate concentration), several authors [3, 11, 19] have suggested the use of an equation analogous to the original Michaelis-Menten model:

$$V = (Vm[E])/(Ke + [E]) \quad , \qquad (3)$$

where $[E]$ is enzyme concentration and Ke is the half saturation constant relative to the substrate sites. This model has previously been used by other authors to study the effect of cellulase concentration on the rates of hydrolysis of crystalline celluloses [11, 19]. We have used it here to calculate the initial reaction rates (Vo) as a function of initial enzyme concentrations [Eo] and hence the values of Vm and Ke from the results of the various kinetic experiments (see Tables V–VII).

TABLE V. Vm (mg/ml·h) calculated from Equation 3 using nonlinear regression methods.

Fabric[a]	TC	TC-CBH I & II	TC-EG I & II
LM	2.70 ± 0.19	0.91 ± 0.02	2.15 ± 0.05
HM	2.74 ± 0.34	1.01 ± 0.04	2.56 ± 0.07
M.LM	3.23 ± 0.07	0.98 ± 0.01	2.72 ± 0.19
R.LM	1.75 ± 0.06	0.79 ± 0.12	1.70 ± 0.07
V.LM	1.63 ± 0.09	0.65 ± 0.02	2.10 ± 0.08
D.LM	1.53 ± 0.06	0.92 ± 0.10	1.92 ± 0.08

[a] Symbols the same as for Table III.

TABLE VI. Ke (mg/ml) calculated from Equation 3 using nonlinear regression methods.

Fabric[a]	TC	TC-CBH I & II	TC-EG I & II
LM	0.14 ± 0.02	0.12 ± 0.01	0.10 ± 0.01
HM	0.10 ± 0.03	0.07 ± 0.01	0.10 ± 0.01
M.LM	0.15 ± 0.01	0.07 ± 0.01	0.14 ± 0.02
R.LM	0.22 ± 0.01	0.25 ± 0.06	0.13 ± 0.01
V.LM	0.11 ± 0.01	0.09 ± 0.01	0.12 ± 0.01
D.LM	0.12 ± 0.01	0.16 ± 0.03	0.11 ± 0.01

[a] Symbols the same as for Table III.

TABLE VII. Vm/Ke (h^{-1}) calculated from Equation 3 using nonlinear regression methods.

Fabric[a]	TC	TC-CBH I & II	TC-EG I & II
LM	19.28 ± 0.19	7.58 ± 0.02	21.50 ± 0.05
HM	27.40 ± 0.34	14.43 ± 0.04	25.60 ± 0.07
M.LM	21.53 ± 0.07	14.00 ± 0.01	19.43 ± 0.19
R.LM	7.95 ± 0.06	3.16 ± 0.12	13.07 ± 0.07
V.LM	14.81 ± 0.10	7.22 ± 0.02	17.50 ± 0.09
D.LM	12.75 ± 0.06	5.75 ± 0.10	17.45 ± 0.08

[a] Symbols the same as for Table III.

Comparisons of TC, TC-CBH I & II, and TC-EG I & II at LM and HM

The Em values in Table III show that at LM, the maximum number of sites available for cellulase adsorption is increased for TC (Em, TC > Em, TC-CBH I & II + Em, TC-EG I & II). This is believed to be due to the fact that a synergistic reaction between CBH and EG components in TC creates more new sites for adsorption (during 30 minutes reaction time) than either of the other cellulase mixtures acting alone. This is despite the fact that corresponding K values (Table IV) indicate much higher equilibrium adsorption levels for the EG-rich and CBH-rich cellulase as compared with TC. We suggest, however, that lower adsorption levels may occur with TC as a result of competitive adsorption and site blocking when all EG and CBH components are present.

At HM, the increased rate of substrate turnover leads, as might be expected, to an increase in the maximum number of adsorption sites. This effect is very much greater for TC-CBH I & II than for the other cellulase mixtures, and is consistent with our previous observation that EG activity increases more than CBH activity at high agitation rates [12]. The much reduced K value (Table IV) for TC-CBH I & II at HM suggests that the desorption rates for EGs increase dramatically as mechanical agitation increases. This may mean that increased EG activity at high agitation results from the

combination of increased availability of sites and increased enzyme "mobility." Since it is random chain scission by EGs that creates new sites for CBH attack and causes most of the fiber fabric strength loss, it is important to understand and control these agitation effects during textile finishing operations in order to deliver the various desired fabric finishes consistently and cost effectively. We can speculate that the increased number of EG-adsorption sites at HM results either from a true increase in fiber surface area or from improved access to more fiber surfaces within the fabric structure. The former is known to occur to some extent because fiber surface fibrillation accompanies EG attack at HM [12]. On the other hand, the relative accessibility to cellulase varieties, at different agitation rates, of fibers at the surface and in the interior of the fabric may also contribute to the observed effects. If the latter is the case, the size of these effects must also be expected to be a function of yarn twist and fabric construction.

The kinetic parameters given in Tables V–VII show that maximum reaction rates are not greatly affected by increased mechanical agitation, but *Ke*, and hence *Vm/Ke*, changes much more markedly for TC-CBH I & II than for TC-EG I & II. This seems to be consistent with the adsorption effects discussed above.

The maximum reaction rates (*Vm*) found for TC and TC-EG I & II are higher than for those for TC-CBH I & II (Table V), and this is consistent with the measured activities of the three cellulase mixtures. The *Vm/Ke* values can be regarded as a measure of catalytic specificity [11], and the higher values for TC and TC-EG I & II compared with TC-CBH I & II would suggest that CBH attack at the ends of cellulose chains is a more specific catalyzed reaction than random cellulose scission by EGs.

Effects on Mercerized Fabrics

Other authors have reported that mercerization of cotton fabrics leads to a somewhat higher rate of cellulolytic hydrolysis [1, 5, 8]. Our results agree with these earlier findings and suggest that the effect is caused by an increased number of available adsorption sites for both EGs and CBHs on the mercerized fabric (see Table III, comparing M.LM with LM). This is reflected in increased *Vm* values for all three cellulase mixtures (Table V) and an apparent increase in the catalytic specificity of EG attack (Table VII, comparing TC-CBH I & II at LM and M.LM). The increased availability of adsorption sites is likely to be related to the change in fiber surface geometry associated with mercerizing. The more cylindrical mercerized fibers could be expected to offer more accessible surfaces than the collapsed and convoluted nonmercerized fibers. An increase in the catalytic specificity of EG attack may be related to changes in cellulose crystallinity and orientation, but no real explanation is offered.

Effects on Dyed Fabrics

We and others have reported that whereas dyeing with direct and reactive dyes reduces the rate of cellulolytic hydrolysis of cellulosic fibers, dyeing with vat dyes has no measurable effect [10, 16]. Our results confirm these findings, and we suggest specific reasons for some the observed effects in terms of the relevant adsorption and kinetic parameters. Although our results relate to one dye only in each of three application classes studied, we believe that the cumulative evidence is sufficient to suggest that these are general effects. This is to be expected, since the nature of dye fiber interactions within an application class is, almost by definition, common to all dyes in the class.

The reactive dye inhibits cellulose hydrolysis by reducing the number of sites available for CBH adsorption (Table III, compare R.LM with LM for TC-EG I & II). This is probably because cellulose chains carrying covalently bound dye molecules cannot easily be accommodated in the catalytic domains of CBH enzyme proteins. These domains are, in fact, now known to be "holes" or "tunnels" that fit precisely around cellulose chain ends [20]. In the course of this work (and previous work with other reactive dyes [10, 16], we also noted that even at very low levels of cotton fabric weight loss, reactive dyed fabric liberated soluble, colored fragments. These were assumed to be cellulose oligomers or single glucose units bearing covalently bound reactive dye molecules. The direct and vat dyed fabrics did not release color into solution until there were fabric weight losses of around 5%. We suggest that these findings also tend to confirm that reactive dyed cotton behaves like other cellulose derivatives that are known to exhibit strong CBH inhibition. It is also notable that the CBH adsorption constant *K* is greatly increased (Table IV, R.LM, TC-EG I & II), which suggests very low mobility of CBHs adsorbed at the scarce cellulose chain ends not modified by reaction with the dye.

The direct dye inhibits cotton hydrolysis by reducing the number of sites available for EG adsorption (Table III, compare D.LM with LM, R.LM, and V.LM for TC-CBH I & II). This is consistent with the view that the large planar direct dye molecules adsorbed on hydrophobic edges of microfibrils block many of the sites that would otherwise be available for EG adsorption.

When vat dyes are oxidized and fully developed by "soaping" at the boil, they form relatively large, insoluble, crystalline aggregates that are firmly trapped

inside fiber, but are not bound at specific sites by strong physical forces. Consequently their presence does not significantly inhibit cellulolytic attack by EGs or CBHs.

Conclusions

We have characterized the activities of TC, EG-rich, and CBH-rich mixtures from *T. reesei* and shown that their activities towards cotton fabrics are influenced by ionic strength and adsorbed ionic species as well as by temperature and pH. Adsorption and kinetic experiments confirm that increasing mechanical agitation favors EG attack by greatly increasing the availability of sites for EG adsorption. It is not clear whether this is a consequence of fiber fibrillation damage or of improved access to fiber surfaces deep within the fabric structure. The enhanced rate of cellulolytic hydrolysis of mercerized cotton and the inhibitory effects on reactive and direct dyed fabrics are explained mainly in terms of increased or reduced availability of adsorption sites for CBHs and EGs.

The implications for textile finishing are far reaching. It is clear that fabric processing history (especially mercerizing and dyeing), fabric construction, and the level of applied mechanical agitation can be as important as the choice of enzyme composition and concentration in determining the consistency and quality of the end result.

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