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Effects of Agitation and Endoglucanase Pretreatment on the Hydrolysis of Cotton Fabrics by a Total Cellulase

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ABSTRACT

We have attempted to clarify the effects of low and high levels of mechanical agitation on the cellulolytic activity of a pure endoglucanase (EG) and a total cellulase mixture (TC) on scoured and bleached cotton fabric, along with the effect of EG pretreatment on subsequent treatments with TC. Methods used to follow the progress of fabric treatments include weight loss, breaking load loss, bending hysteresis, and the production of soluble reducing sugars and reducing end groups in the fibers. The results show that agitation rate affects the mechanism of cellulolytic attack, and that this has implications for delivering desired finishing effects.

In recent years, there has been increasing interest in the use of enzymes to produce specific finishing effects on a variety of textile substrates. While this interest stems in part from the absence of environmental problems associated with enzymatic processes and effluents [3, 11], the impetus for recent activity also derives from advances in biotechnology that have facilitated the production of new enzymes with more specific catalytic activities and better defined activity profiles than those previously available [3, 11].

Several different kinds of cellulase are now available to treat cellulosic textiles. Finishing denims to produce the fashionable stone-washed appearance remains the most widely used application [12], but other processes that improve fabric appearance (by removing fuzz-fibers and pills) or deliver softening benefits are also being introduced [1, 3]. Increasing use is also being made of cellulases in domestic fabric washing products, where they are claimed to aid detergency [13] as well as remove damaged fibrillar material from fiber surfaces. This improves fabric appearance, color brightness, and softness [1].

Cellulase enzymes have specific catalytic action on the hydrolysis of the 1,4-β-D glycosidic bonds in cellulose polymers. Natural cellulases are secreted by various fungi and bacteria as complex mixtures of three major kinds: endoglucanase (EG, EC 3.2.1.4), cellobiohydrolase (CBH, EC 3.2.1.91), and cellobiase (EC 3.2.1.21) [9, 14]. These natural mixtures can be described as "total crude" cellulases (TC). A general model [9] for the action of the different components of TCs is, first, that EGs cause random hydrolytic chain scission at the most accessible points of cellulose chains, producing one new reducing end group and one new nonreducing group at each point of scission (this is often known simply as "endo" activity). Second, CBHs split cellobiose from the nonreducing ends of cellulose chains, leaving a new nonreducing end group that can be further attacked by CBH action; it therefore proceeds in a stepwise manner along cellulose chains (this is also known as exoglucanase activity or simply "exo" activity). Third, cellobiase also hydrolyzes cellobiose to glucose.

The development of recombinant DNA techniques has made it possible to manipulate the genes of cellulase-secreting fungi [14]. Using these techniques, derivative fungal strains can be produced that secrete new cellulase compositions, in which one or more components are enriched or deleted [14]. Studies with such engineered cellulase mixtures (and with pure components separated from them) have led to an improved understanding of the mechanism of cellulolytic hydrolysis.

It is now apparent that the general model outlined above is an over-simplification. It appears, for example, that CBHs may also exhibit some endoglucanase activity [2, 9], and they may catalyse hydrolysis at re-

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ducing as well as nonreducing end groups [2]. The expected synergy between the major EG and CBH components has been confirmed, but studies of cotton fabrics have noted that increasing mechanical action appears to have a relatively greater effect on EG activity [4, 5]. This could have important implications for the consistent delivery of specific textile finishing effects, because cellulase finishing may be applied using a variety of dyeing machines and methods that involve very different modes and levels of agitation. These range, for example, from zero agitation in the pad-batch process, through low levels of agitation on jigs and intermediate levels on winches, to much more vigorous action in jet machines and rotating drum washers.

It is essential to know which process variables are the most important in determining the cost effective delivery of a particular finishing effect. These variables may be considered to include different combinations of individual cellulase components (used either together or sequentially), as well as the choice of application method, and the more usual operating variables such as enzyme concentration, liquor-to-cloth ratio, treatment time, temperature, pH, and machine "speed" or agitation level. Pure EGs (or cellulase mixtures rich in EGs) are preferable for achieving the ageing effect on denims [8], and this effect is best obtained in machines that provide a vigorous beating action [3, 4, 5, 12]. A careful balance between cellulase activity and mechanical action is required to achieve efficient fuzz-fiber and pill removal without excessive fabric strength and weight loss [1, 3, 4]. The effectiveness of cellulase finishing treatments is also influenced by the previous processing history of the fabric [4, 6]. The rate of cellulolytic attack is increased by processes such as scouring, bleaching, and mercerizing, which improve the accessibility of cellulose surfaces to the enzyme, but it is retarded by reactive dyeing and other processes, which chemically modify the cellulose structure [4, 6].

In our current study, we attempt to clarify the effects of low and high levels of mechanical agitation on the cellulolytic activities of a pure EG and a TC on scoured and bleached cotton fabric. In addition, since EG treatments may be expected to create new sites for cellulolytic attack by CBHs, we also investigate the effect of EG pretreatment on the rate of subsequent hydrolysis by a TC with a view to the design of more efficient fabric processing regimes.

Experimental

Cellulases were supplied by Novo Nordisk A/S. The TC was a commercial preparation, Cellusoft L, and the EG was a development sample supplied under the code name SP492-PPC 3617. (The names of these materials are given for information only and their mention does not constitute a recommendation.) We determined the activity of these cellulases on various cellulosic substrates using the methods described below. All treatments were involved 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 100g/m^2 weight.

Enzyme Activity

We determined the activity of cellulases on carboxymethylcellulose (CMC) and phosphoric acid swollen Avicel (PASA) by means of the increase in concentration of the liberated soluble reducing sugars using methods similar to those previously described by Evans *et al.* [10]. Activity on cellobiose was determined using the glucose-oxidase assay method [10], and activity on scoured cotton fabric was determined by measuring fabric weight loss and the concentrations of liberated, soluble reducing sugars. These were obtained by means of formation of the colored, cuprous neocuproine complex (see other methods below). In all cases, cellulase activity was measured at 50°C. TC hydrolyses were done at pH 4.8 (0.5 *M* acetate buffer) and EG hydrolyses at pH 7.0 (0.5 *M* phosphate buffer).

Fabric Treatments

Low Agitation

For the EG treatment/pretreatment, fabric samples (10 g) were placed in the stainless steel pots (500 ml) of a Linitest machine. To these were added either pH 7.0 buffer solution (100 ml) or a solution (100 ml) of EG (1 g/l) in the same buffer. The pots were rotated in the Linitester at 65 rpm, with the water bath set at 50°C for periods of 20, 40, 60, and 120 minutes. The enzyme reactions were quenched (in solution and on the fabric) by adding 10% (w/v) sodium carbonate solution (50% dilution of the liquor). Fabric pieces were rinsed thoroughly, dried, and conditioned at 20°C and 65% RH. Treatment solutions were retained for determining soluble sugars.

For the TC treatment, untreated and EG pretreated fabric samples (10 g) were again placed in the stainless steel pots of the Linitest machine. To these were added (100 ml) aliquots of TC (1 g/l) in pH 4.8 buffer solution. The pots were rotated in the Linitester at 65 rpm at 50°C for TC treatment times of 30, 60, and 120 minutes. The treatments were quenched and the fabrics were rinsed, dried, and conditioned as before.

High Agitation

Fabrics were treated in a manner identical to those described above for both EG and TC, except that a high level of mechanical agitation was achieved by adding twelve stainless steel discs to each Linitest pot for every treatment. (These discs are supplied with the Linitest machine to provide an increased level of mechanical agitation in various standard fastness test methods.)

OTHER METHODS

Fabric weight loss was determined by the difference in the weights of fabric samples before and after treatment and always after conditioning for 24 hours at 20°C and 65% RH. Soluble reducing sugars were determined by boiling the treatment solutions with an alkaline neocuproine-Cu (II) complex. This produced the colored Cu (I) complex, the concentration of which was obtained by means of its absorbance at 475 nm using a Hitachi U-2000 UV-vis spectrophotometer.

The reducing power of end group contents of fabric samples was determined by boiling 100 mg of the cotton sample in an alkaline solution of neocuproine-Cu(II) complex. This produced the Cu(I) complex (in solution), the concentration of which was obtained as described above.

Fabric strength was measured (at 20°C and 65% RH) as % loss in breaking load of cellulase treated samples relative to untreated fabric, using an Instron model 4204. Samples (20 cm × 3 cm, 15 cm gauge length) were tested in the warp direction only. Fabric bending hysteresis was measured (at 20°C and 65% RH) using a KES-FB2 instrument from Kato Tech. Co. Ltd. Samples were tested in the warp direction only.

Fiber crystallinity indices were obtained with the x-ray diffraction method described by Chidambareswaran *et al.* [7]. X-ray diagrams were obtained using a Philips analytical PW1710 diffractometer and an x-ray tube using Ni filtered Cu K_α radiation. The angular limits were 10° and 30°. Scanning electron microscopy photographs (Leica Cambridge Stereoscan 360) were obtained after 2 minutes of gold metalization (Bio-Rad SC 502) of the fabric samples.

Results and Discussion

Table I shows the measured activity of EG and TC. The TC hydrolyzed all substrates to soluble sugars, indicating the presence of all three major cellulase components (EGs, CBHs, and cellobiases) [5, 9, 19]. The EG had the expected greater relative activity on CMC than on PASA, and no measurable activity on cellobiose and cotton. This suggests that the EG sample had purely "endo" activity [5, 9, 19], which is not measurable on crystalline cellulose as weight loss or by liberating soluble sugars.

EFFECTS OF EG TREATMENTS

Low Agitation

There was no measurable cotton weight loss resulting from the low agitation treatment with EG (Figure 1a), and no soluble sugars were detected in solution (Figure 2a). This is consistent with the results of the enzyme activity measurements described above. There was, however, good evidence of EG activity on the cotton fibers from their increased reducing group content (Figure 3a) and from the loss in fabric breaking load (Figure 4a). It is interesting to note that both of these properties appear to have reached an almost constant value after the first 20 minutes of treatment. We suggest that this corresponds to a saturation level of enzyme adsorption (and hydrolytic attack) on the accessible surface area of the fibers, and that the low agitation in the system is insufficient to detach the relative long lengths of randomly hydrolyzed cellulose oligomers from the fiber surface. In this case, no further hydrolytic

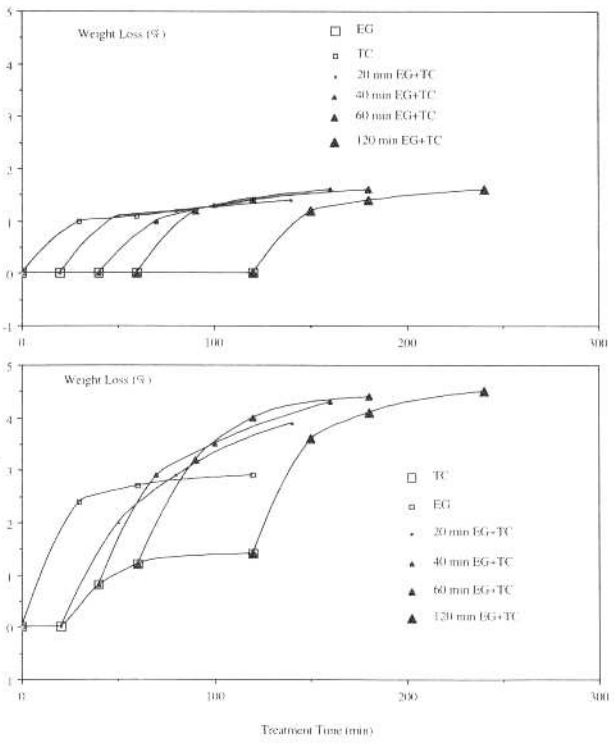

FIGURE 1. Effects of EG and TC treatment times on fabric weight loss under conditions of (a, top) low and (b, bottom) high levels of mechanical agitation.

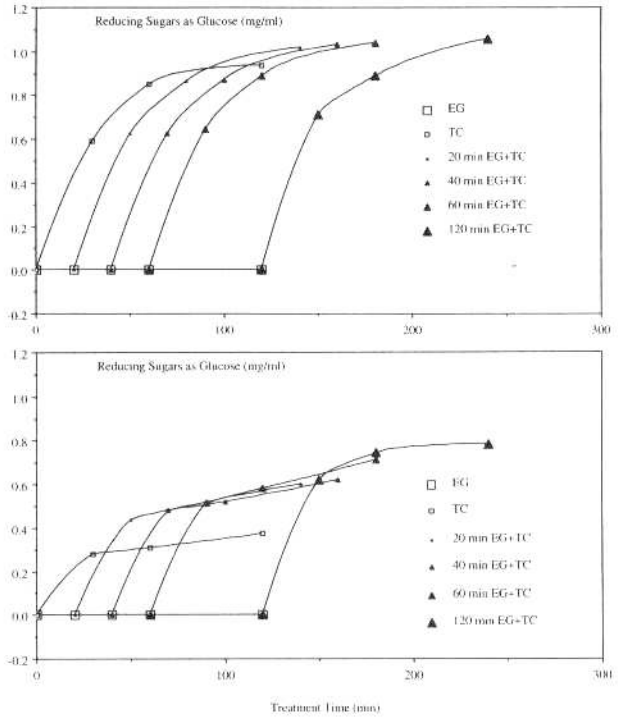

FIGURE 2. Effects of EG and TC treatment times on the release of soluble reducing sugars in conditions of (a, top) low and (b, bottom) high levels of mechanical agitation.

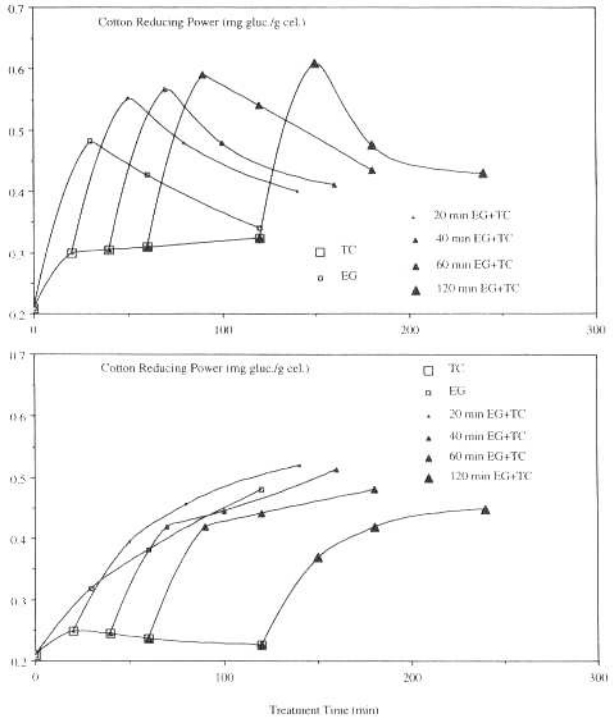

FIGURE 3. Effects of EG and TC treatment times on the concentration of reducing end-groups in cotton fabrics under conditions of (a, top) low and (b, bottom) high levels of mechanical agitation.

attack by EG would be possible. Further work is, necessary, however, to confirm whether this is in fact the case.

High Agitation

The high agitation EG treatments, lasting 40 minutes or more, caused increasing cotton weight loss up to about 1.5% after 120 minutes (Figure 1b), but again this was not accompanied by the liberation of soluble reducing sugars (Figure 2b). All weight loss during these treatments was accounted for by an insoluble deposit of microfibrillar cotton debris in the reaction pots. Identical high agitation treatment in the absence of enzyme caused less than 0.2% cotton weight loss after 200 minutes, and this did not produce any cellulose debris. We can therefore conclude that the combination of EG treatment with high agitation levels causes microfibrillar material to be torn away from the fiber surface. This exposes fresh cellulose surfaces, which are then subject to further EG attack. This may also be appreciated by reference to Figure 6: 6a shows no significant damage to the cotton fiber surface after 120 minutes EG treatment at low agitation, whereas Figure 6e shows the torn fiber surfaces after similar treatment at high agitation, and Figure 6d confirms that high agitation without EG does not cause this fiber damage.

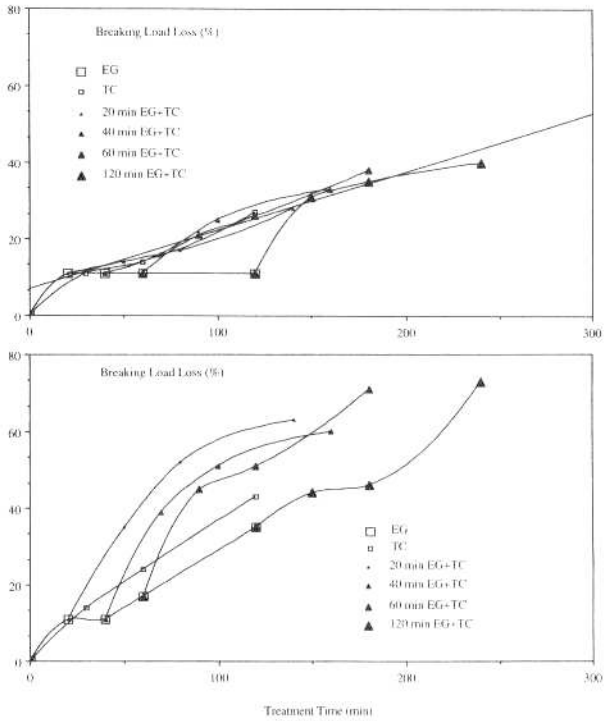

FIGURE 4. Effects of EG and TC treatment times on the breaking load loss % of cotton fabrics under conditions of (a, top) low and (b, bottom) high levels of mechanical agitation.

After high agitation treatments, the measured concentration of reducing end groups in the fiber (Figure 3b) was consistently lower than after low agitation treatments (Figure 3a) and also tended to decrease with treatment time. We believe that this resulted from the continual removal of microfibrillar fragments, which may be expected to have a higher reducing group content than the bulk of the fibers.

Increased fabric breaking load loss with treatment time (Figure 4b) provided evidence for progressive EG attack on the fibers. The breaking load loss of about 35% after 120 minutes treatment was accompanied by only about 1.5% weight loss. This is typical of pure endo activity: random chain scission is expected to have the maximum effect on reducing polymer average chain lengths, thereby dramatically reducing fiber and fabric tensile strength.

EFFECTS OF TC TREATMENTS

Low Agitation

The TC treatments at low agitation produced fabric weight losses of up to 1.5% after 120 minutes treatment from both untreated and EG pretreated fabrics, regardless of EG pretreatment time (Figure 1a). It was apparent from the rapid rise in the concentration of soluble reducing sugars (Figure 2a) that this material loss was due mainly to the exoglucanase activity of the CBHs present in TC.

The concentration of reducing end groups in the fibers produced by TC treatment increased to a maximum value and then declined, irrespective of EG pretreatment time (Figure 3a). We believe this is due to the endo activity of TC producing (further) random chain scission of cellulose molecules at the fiber surfaces, before partially hydrolyzed material (of high reducing group content) is dissolved away as cellobiose by the CBH activity of TC. It appears from the magnitude of the initial increase in reducing group content, caused by TC, that the endo activity of this enzyme complex is much greater than that of the pure EG. This is consistent with the measured activities of the two enzymes given in Table I.

The fabric breaking load loss (BLL) after low agitation TC treatments was approximately proportional to total treatment time by EG + TC (Figure 4a), despite the fact that the EG pretreatments caused about 10% BLL regardless of treatment time. This suggests that the longer EG pretreatments caused an accelerated rate of BLL in subsequent TC treatments, and it is the only evidence to suggest that low agitation pretreatments with EG can potentiate subsequent hydrolyses by TC. Remember, however, that in these experiments, the pure EG activity was low compared with the endo activity of the TC used in the subsequent treatments (Table I).

TABLE I. Measured enzyme activity (see text for individual hydrolysis conditions and assay methods).

Substrate/Enzyme	EG	TC
Cellobiose[a]	0 U/g	0.75 U/g
CMC[b]	73 U/g	159 U/g
PASA[b]	28 U/g	361 U/g
Cotton[bc]	0 U/g	51 U/g
	0%	1.33%

[a] One unit (U) liberates 2 μmol of glucose per minute minute. [b] One unit (U) liberates 1 μmol of reducing sugars (as glucose) per minute. [c] Cotton fabric weight loss (%) caused by treatment for 1 hour with 1 g enzyme under the conditions described.

High Agitation

High agitation treatments with TC gave a limiting fabric weight loss of about 3% in addition to that caused by EG pretreatments (Figure 1b); this compares with about 1.5% in low agitation treatments, but the concentration of reducing sugars found in high agitation solutions was reduced to about one third of the level for treatments with low agitation (Figure 2). This may be partly explained if the mean chain length of the leaving oligomers is much greater in the high agitation treatments. We confirmed this to the extent that much of the lost material was in the form of the insoluble microfibrillar fragments mentioned above. Nevertheless, the low concentration of soluble sugars implies that "exo" activity of CBHs in the TC must have been reduced in the high agitation treatments. We may be able to explain this if competitive adsorption between EGs and CBHs results in preferential adsorption of EGs at higher agitation rates. Both the progressive increase in the concentration of reducing end groups in the fabric (Figure 3b) and the marked increase in BLL caused by TC treatments at high agitation (Figure 4b) tend to support this hypothesis, since both of these effects are characteristic of endo rather than exo activity.

Pretreatment with EG clearly increased the exo activity of TC in subsequent high agitation treatments (Figure 2b), but the concentration of liberated soluble sugars was still lower than that produced in the low agitation treatments (Figure 2a). BLL results (Figure 4b) indicate an increased risk of serious fabric damage in sequential treatments of this sort. There would, however, appear to be some scope for developing processes in which a high agitation EG treatment is followed by a low agitation treatment with a TC or a CBH-rich cellulase.

FABRIC BENDING HYSTERESIS AND SEM

Low agitation EG treatments produced little change in fabric bending hysteresis (2HB) (Figure 5a), whereas high agitation EG treatments caused a progressive increase in 2HB (Figure 5b). The latter is believed to result from increased interfiber friction, caused by the combination of EG action and high levels of mechanical agitation raising a stubble of fibrillar material on the fiber surface. Evidence for this is illustrated in Figure 6, where 6a shows smooth fibers after 120 minutes low agitation treatment with EG, but 6e shows seriously roughened fibers after similar EG treatment at high agitation.

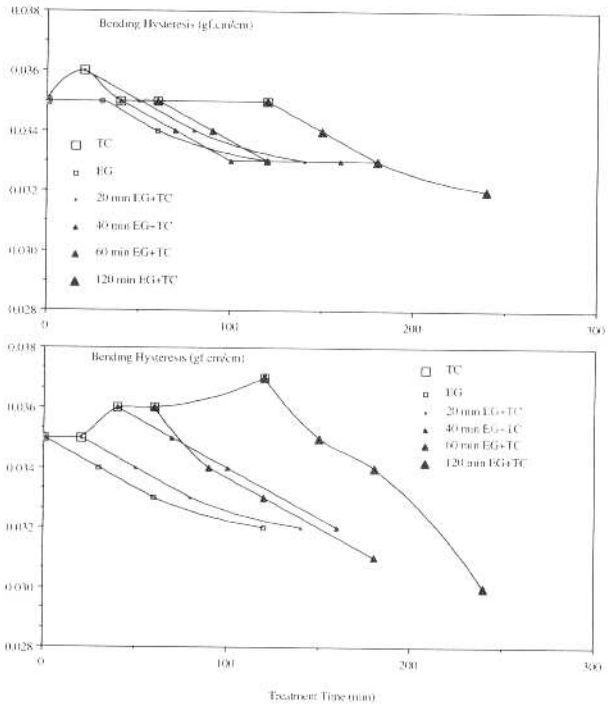

FIGURE 5. Effects of EG and TC treatment times on the bending hysteresis of cotton fabrics under conditions of (a, top) low and (b, bottom) high levels of mechanical agitation.

Treatments with TC in both low and high agitation regimes caused reductions in 2HB. In the cases where TC treatment followed EG pretreatment with high agitation, the reduction in 2HB was clearly attributable (at least in part) to reduced interfiber friction caused by "biopolishing"—the enzymatic removal of the stubble of damaged material from fiber surfaces (compare Figure 6e with 6g). In all TC treatments, however, it is also possible that the removal of small amounts of cellulosic material from cotton fibers significantly reduces fiber bending stiffness, particularly if cracks are formed in the fiber primary and secondary wall (see Figure 6b). Such a mechanism may contribute to the observed reductions in fabric bending hysteresis, especially where there is little evidence of a biopolishing effect.

In general, fabrics with low interfiber friction (low 2HB) are perceived to be soft [15]. It will be apparent from our results that EG treatments with high levels of mechanical agitation may be expected to make fabrics feel harsher, whereas all TC treatments may be expected to provide fabric softening benefits.

FIBER CRYSTALLINITY INDEX

There has been discussion over the years as to whether cotton cellulose should be regarded as a two-phase polymer composed of crystalline and amorphous regions, or as a one-phase polymer composed of crystalline regions having varying degrees of imperfection and accessibility [17, 18]. Sagar suggested that in a two-phase structure, cellulolytic attack would remove the more accessible amorphous material preferentially [18], and the "average" crystallinity of the structure should therefore be expected to increase. In a one-phase structure, cellulolytic attack should occur at the most accessible crystal surfaces, and should produce no change in overall crystallinity and a constant kinetic order during hydrolysis [18].

The results of the fiber crystallinity measurements for our work, shown in Table II, indicate no changes in fiber crystallinity, regardless of cellulase type or treatment agitation regime. These results therefore tend to support the idea of a one-phase cellulose model, and do not reveal anything further of the mechanism of cellulolytic hydrolysis of cotton. Previous work [16] has produced similar results for various other cellulases and cellulosic substrates. Furthermore, the hydrolysis of cotton cellulose by concentrates of *Trichoderma viride* are first-order with respect to the substrate [18].

TABLE II. Crystallinity index of cotton fabrics.

Treatment	Low agitation	High agitation
None	0.83	0.83
EG (120 minutes)	0.82	0.82
TC (120 minutes)	0.83	0.83
EG (120 minutes) + TC (120 minutes)	0.83	0.83

Conclusions

At low agitation levels, pretreatment with EG did not have a major influence on the course of subsequent

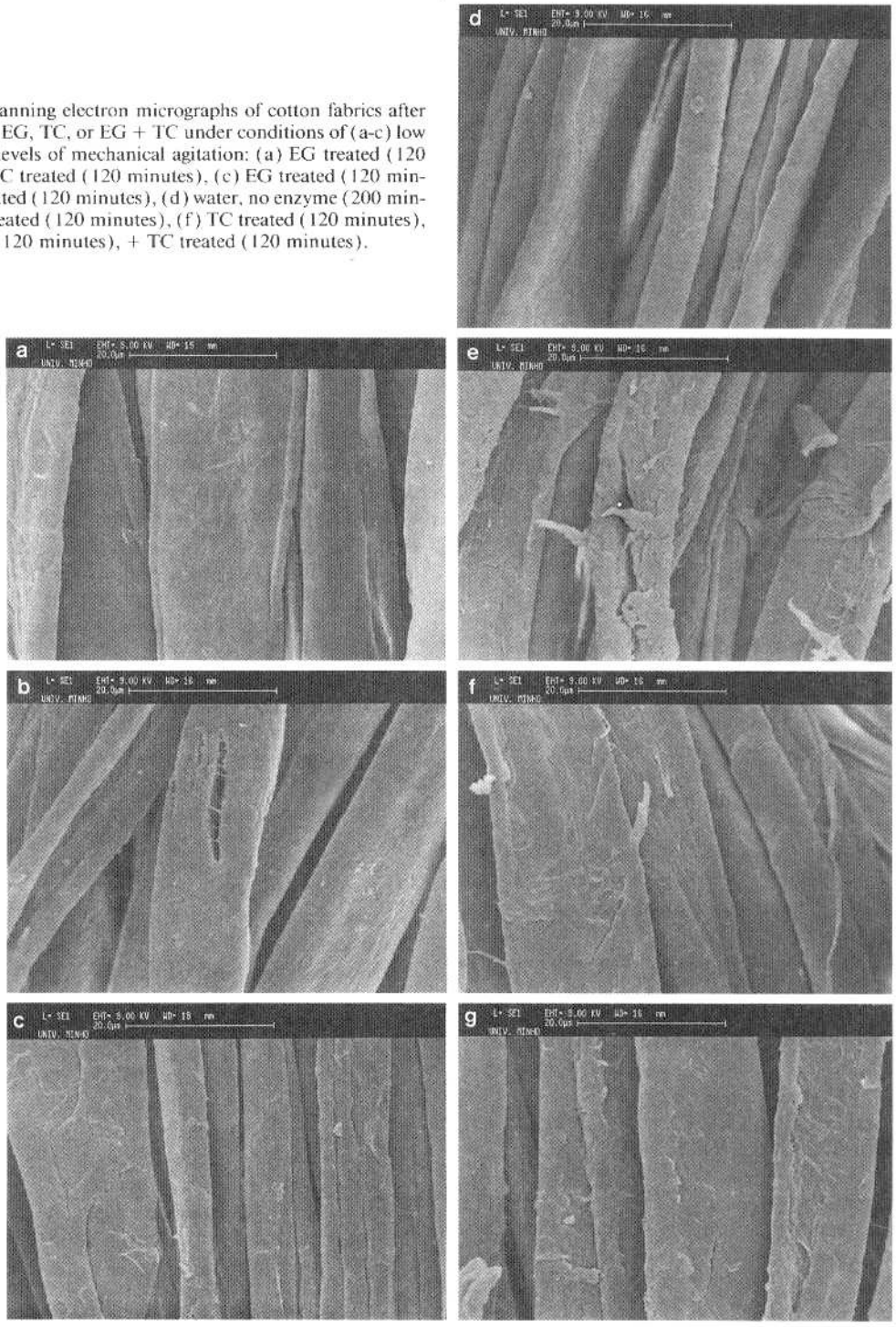

FIGURE 6. Scanning electron micrographs of cotton fabrics after treatments with EG, TC, or EG + TC under conditions of (a-c) low and (d-g) high levels of mechanical agitation: (a) EG treated (120 minutes), (b) TC treated (120 minutes), (c) EG treated (120 minutes), + TC treated (120 minutes), (d) water, no enzyme (200 minutes), (e) EG treated (120 minutes), (f) TC treated (120 minutes), (g) EG treated (120 minutes), + TC treated (120 minutes).

TC treatments. This may, however, have been due to the relative endo activity levels of the EG and TC used in this work. At high agitation levels, EG activity increased while CBH activity of TC decreased. As a consequence, there was increased risk of serious fabric strength loss. There may, however, be further scope for developing sequential processes in which high agitation EG treatments are followed by low agitation treatments with TC or CBH-rich cellulases. The development of sequential treatments with carefully selected enzyme components should therefore not be ignored in future work.

More importantly, since we have shown that the level of mechanical agitation applied during cellulase finishing treatments affects the mechanism of cellulolytic attack, the choice of processing method must be expected to be a source of variability in the delivery of desired cellulase finishing effects. These findings suggest a need for further work, which should lead to more predictable and better controlled cellulase finishing processes.

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