24 The treatment of cotton cellulose with *Trichoderma reesei* engineered cellulases

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1-INTRODUCTION

Controlled enzymatic hydrolysis can provide ecologically acceptable routes to finishing cellulosic textiles (1). The most widely used application is the replacement of the stone washing process to produce the fashionable aged appearance of denims. Other cellulase treatments are used to improve the appearance of cotton fabrics by removing fuzz fibre and pills from the surface. Such processes also modify the fabric mechanical properties in ways which lead to the perception of improved handle, particularly of improved softness (2).

Increasing use is being made of cellulases in domestic fabric washing products where they are claimed to aid detergency (3), as well as removing damaged fibrillar material from cotton fibre surfaces. This improves fabric appearance, colour brightness and softness (1).

Cellulase enzymes have a specific catalytic action on 1,4-β-D glycosidic bonds of the cellulose polymer, which apart from 6-8% of moisture, is essentially the sole constituent of scoured and bleached cotton. Cellulase, by definition, consists of a complex mixture of three major enzyme types: endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91) and cellobiases (EC 3.2.1.21) (4). A general model for action of these enzyme components on cotton cellulose is that the endoglucanases (EGs) cause random hydrolytic chain scission at the most accessible points of long cellulose polymers, while the cellobiohydrolases (CBHs) split cellobiose from the "non reducing end" of cellulose molecules. The cellobiase hydrolyzes cellobiose to glucose. Improvement in purification and analysis techniques have lead to the isolation and study of the pure components and new models have been proposed. It is now clear that the mode of action of the pure cellulase components cannot be classified simply as "endo" or "exo" in type (5). A synergism between the different components has been observed, but the detailed mechanism of their action is not yet fully understood (5).

The cellulolytic complex of *Trichoderma reesei* is one of the most extensively investigated fungal enzyme systems (5). It is known to contain at least one cellobiase, CBH I, CBH II, EG I and EG II. The genes of these hydrolases can be manipulated such that some activities are deleted to produce new cellulase combinations (6). Furthermore it has been reported in recent literature (7, 8) that two more cellulase components known as EG III and EG V are always present in the crude mixtures from *Trichoderma reesei.*

In the present work, cotton degradation was studied after short (as made in textile applications) and extended periods of cellulase hydrolysis. Comparisons were made between the effects of crude cellulase mixture from *Trichoderma reesei* (TC), and mixtures in which the activities of EG I and EG II (C-EGs) or the activities of CBH I and CBH II (C-CBHs) had been deleted.

2-EXPERIMENTAL

Cellulases: All enzyme samples are from Primalco Ltd., Rajamäki, Finland. These were: CE 883042 (Total crude), CE 519/92 (Crude-EGs) and CE 523/92 (Crude-CBHs).
Enzyme Activity: The activities per gram of crude were measured towards carboxymethylcellulose (CMC), phosphoric acid swollen Avicel (PASA) and cellobiose, at pH=5, as described previously (9). The activity towards scoured cotton fabric was also determined by measuring weight loss.
Cotton Samples: All samples used were 100% cotton fabrics after industrial scouring and bleaching.
Treatments:
Short: The fabrics were treated with the enzyme (dilution factor, 1/500) using the ratio: 1g of fibre to 20 ml of bath; buffer pH = 4.8 (acetate, 0.5 M), temperature 50°C. The fabrics were treated during 30, 60, 120 and 240 minutes. The treatments were stopped by addition of a solution of sodium carbonate (10 %). The fabrics were washed after treatment with hot and cold water. The treatments were carried out in the stainless steel pots of a Linitest machine rotating at 65 rpm.
Long: The fabrics were treated with the enzyme (dilution factor, 1/60 for the Crude-CBHs and 1/120 for the Total crude and Crude-EGs) using the ratio: 1g of fibre to 50 ml of bath; buffer pH=4.8 (acetate, 0.5 M), temperature 50°C. The fabrics were treated without agitation for 3, 6 and 13 days in a beaker inside an incubator. The fabrics were washed after treatment with hot and cold water.
Weight Loss was determined by weighing the samples before and after treatment, after conditioning for 24 hours at 20°C and 65 % of relative humidity.
Mean Chain Length: Mean chain lengths were determined by the weight difference taken from weight loss calculations and measuring in solution the reducing ends of the leaving sugars as cuprous neocuproine complex in alkaline media at 95°C.
Cotton Reducing Power: The reducing ends of the cotton fibres were also quantified via the cuprous neocuproine complex in alkaline media at 95°C.
Viscosity measurements: Fluidity and specific viscosity were measured as described (10), on cuproethylenediamine (CED) solutions of cotton cellulose.
Breaking Load Loss (%) was measured relative to the untreated fabric in an Instron machine, model 4204.
Bending Hysteresis of the Fabrics was measured on KES-FB2 from Kato Tech. Co, Ltd.
Crystallinity Index by X-ray diffraction measurement was obtained by the method described by Chidambareswaran and others (11). The X-ray diagrams were obtained using a Philips Analytical PW1710 diffractometer with a X-ray tube using Ni filtered Cu K_α radiation and the limits were 10° and 30°.

Scanning Electron Microscopy Photographs (Leica Cambridge Stereoscan 360) were taken after 2 minutes of gold metalization (Bio-Rad SC 502).
Environmental Scanning Electron Microscopy (Electron Scan, Mod 3) A video-tape was recorded during the drying of cotton fibres, which had never been dried after the cellulase treatment. The pressure of the chamber during drying was brought down from 6 Torr to 2,5 Torr.

3-RESULTS AND DISCUSSION

The measured activities of C-CBHs towards CMC and of C-EGs towards PASA were greater than those of TC (tab. 1). These results illustrate the expected increments in classical endo and exo type activities for C-CBHs and for C-EGs respectively (12). While TC was found to have lower measured activity than C-EGs towards cellobiose, CMC and PASA, it caused consistently greater cotton weight loss than C-EGs in both long and short treatments (tab. 1 and fig. 1). This apparent contradiction shows that care should be exercised in predicting cellulase activity on cotton, from data obtained using other forms of cellulose or its derivatives. The result also points to the importance of synergy between the various components in the hydrolysis of cotton cellulose.

TABLE 1 - Enzyme Activity

Substrate \ Enzyme	Total crude	Crude - CBHs	Crude - EGs
Cellobiose 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
Weight Loss-Cotton	1,40 %	0,47 %	1,05 %

a) - 1 Unit yields 2 µmol of glucose per minute in the cellobiase hydrolysis
b) - 1 Unit yields 1 µmol of reducing sugars as glucose per minute

The deletion of CBH I and CBH II activity from the total crude mixture dramatically reduced the rate of cotton weight loss (fig. 1) thus confirming the importance of exo type activity in solubilization of the polymer. The deletion of EG I and EG II activity also caused some reduction in the rate of cotton weight-loss, and this is expected from the synergy between endo and exo type activity. Nevertheless, it is clear that C-EGs and TC can both bring about complete dissolution of cotton cellulose, albeit in somewhat longer reaction times, as shown by the SEM photos after 6 days of degradation (fig. 2a). The surprisingly high activity of C-EGs may possibly due to the previously reported synergy between the two CBH components (13) and, or, to some endo activity in our C-EGs. The latter may be accounted for either by remaining EGs such as EG III and V or by some endo characteristics of the CBHs themselves (5, 14).

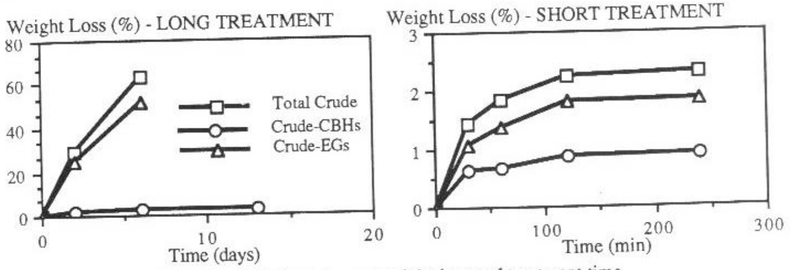

Figure 1 - Relation between weight-loss and treatment time.

In the short hydrolysis, the relative activity of C-CBHs is higher and the relative activity of C-EGs is lower, when compared with the longer hydrolysis. This seems to be due to the rotation of the reactor where the hydrolysis takes place. The agitation is known to increase the weight loss (15) and to have a synergistic action with an endo type cellulase (16). However the main structure of the fibres remains unchanged (fig. 2b).

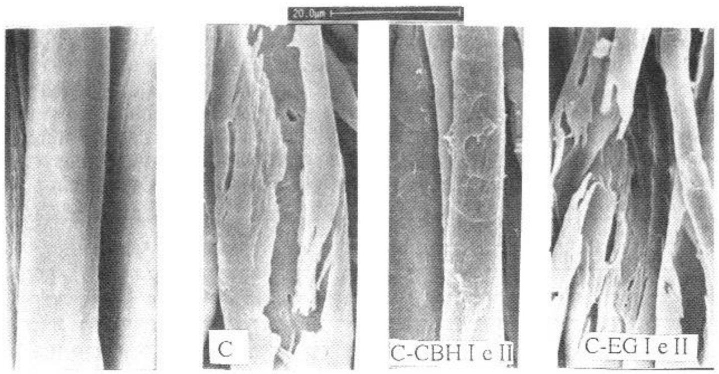

Figure 2 a) Scanning electron microscopy photographs of long treated fibres

Figure 2 b) Scanning electron microscopy photographs of short treated fibres

SEM photos (fig. 2) showed that the short treatment caused the changes only at the fibre surface, while the long treatments also affected the internal structure of the fibre.

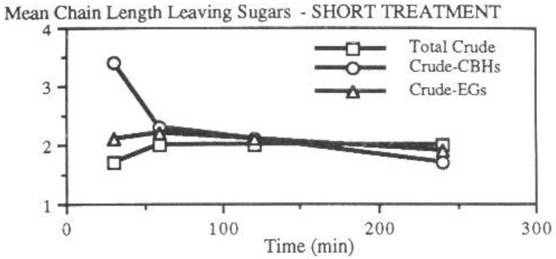

Figure 3 - Relation between mean chain length of the leaving sugars and treatment time.

In treatments with C-CBHs, the mean chain length of the leaving sugars (fig. 3) decreased from 3.4 (30 min) to 1.7 (240 min) showing endo character when compared with the other mixtures where the mean chain length was about 2 throughout. The results show that the soluble cellooligosaccharides resulting from TC and C-EGs hydrolysis are readily broken as they are produced to a mean length of 2, while those resulting from C-CBHs are slowly broken. The slow decrease of the mean chain length of the soluble cellooligosaccharides caused by C-CBHs is believed to be due to the lower cleavage frequencies of EGs compared with those of CBHs (14), on the soluble cellooligosaccharides. The low cellobiase activity of these cellulase mixtures is also reflected in the results.

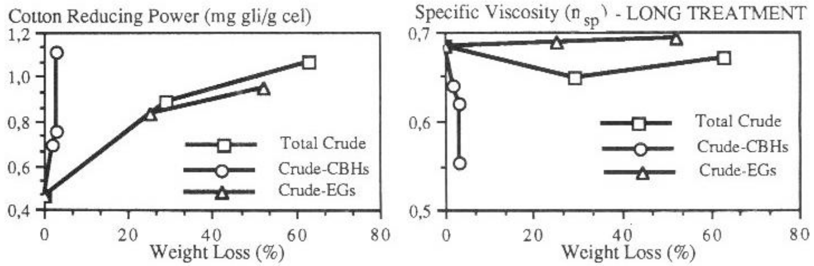

Figure 4 - Relation between cotton reducing power and between specific viscosity with weight-loss

In spite of causing low weight loss, the C-CBHs enzyme produced higher concentrations of terminal reducing groups in the cotton fibres and a large decrease in the viscosity of their CED solutions (fig. 4). TC and C-EGs also produced appreciable quantities of terminal reducing groups but the viscosity of CED solutions of the treated celluloses changed only slightly, even at a weight loss greater than 50 %. These results confirm the increased endo type activity of C-CBHs in causing random cellulose chain scission, (hence a large reduction in CED solution viscosity) and the sequential nature of exo type activity, (hence little change in mean polymer chain length or CED solution viscosity).

These effects are further illustrated in figure 5, where an increasing slope of the plot of cotton fluidity versus cotton reducing power indicates increasing randomness of cellulolytic attack. Thus C-CBHs is shown to cause the most random hydrolysis and C-EGs to cause the most localized attack, with TC having an intermediate effect. While the randomness of cellulolytic action of different cellulases is commonly measured in this way by using CMC as a soluble substrate (17) these relationships have not previously been reported for cotton cellulose.

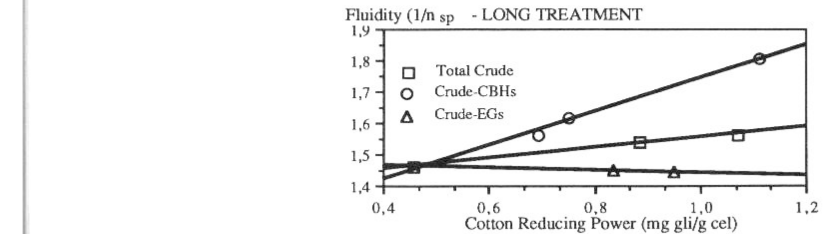

Figure 5 - Relation between cotton reducing power and specific viscosity

It is interesting to note that the relative randomness or localization of cellulase action on cotton and the extent of the produced damage (fig. 2) did not change the measured crystallinity of the fibre (tab. 2). A similar result has previously been observed for complete cellulases (18). Thus the present work tends to confirm the view that the action of cellulase is not confined initially to non-crystalline regions, irrespective of the cellulase components present. It has also been reported (19) that the hydrolysis of cellulose by cellulase concentrates of *Trichoderma viride* is first order with respect to substrate. This suggests uniform reactivity of (crystalline and amorphous) cellulose and therefore implies that no change in crystallinity should result from cellulase hydrolysis.

TABLE 2 - Crystallinity Index (Weight-Loss)

Untreated	TC 240min	C-CBHs 240min	C-EGs 240min	TC 6 days	C-CBHs 6 days	C-EGs 6 days
83% (0%)	83% (2,3%)	83% (0,9%)	83% (1,8%)	83% (63%)	83% (3%)	81% (52%)

Cellulase hydrolysis reduced the tensile strength cotton fabrics as shown in figure 6. Recently a possible relation between loss in tensile strength and endo activity has been reported (16). The present results support this to the extent that C-CBHs caused much greater strength loss at low weight loss than the other cellulase mixtures (fig. 6). On the other hand C-EGs did not show reduced strength loss for a given weight loss in comparison with TC. This may however be due to the effects of the residual EGs (III and V) in the present C-EGs sample.

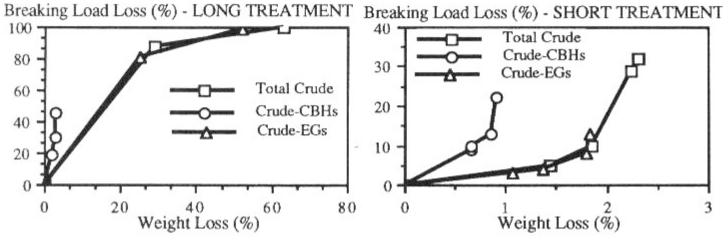

Figure 6 - Relation between breaking load loss and weight-loss

We have observed before (16) that for short cellulase treatments the changes in bending hysteresis of cotton fabrics, related to internal friction of the fibrous assembly, are due to the formation of microfibrils on (or their removal from) the fibre surface. The small changes in bending hysteresis, for the short treatment, are consistent with what can be seen in SEM photos (fig. 2b). The large decrease in bending hysteresis for higher weight loss (long treatments) observed here is mainly due to the reduction in the number of fibres in a yarn and reduction of its diameter, as shown by SEM photos (fig. 2a). In as far as low bending stiffness/hysteresis is correlated with perceived softness, TC and C-EGs would be preferred over C-CBHs for their cotton fabric softening effects. This is consistent with a previously reported observation (16) that endo activity with high mechanical action led to the formation of microfibrils on fibre surface and caused increased inter-fibre friction, and hence an increase in bending hysteresis.

TABLE 3 - Bending Hysteresis (gf.cm/cm)

Untreated	TC 240min	C-CBHs 240min	C-EGs 240min	TC 2 days	C-CBHs 2 days	C-EGs 2 days
0.050	0.043	0.053	0.045	0.015	0.048	0.019

Examination of the wetting (swelling) and drying (collapsing) behavior of untreated and cellulase treated cotton fibres by ESEM revealed that the fibre twisting and retraction behavior in untreated fibres is absent after long cellulase treatments. It is therefore postulated that a cellulase treatment may be beneficial to achieving stable, fully-relaxed dimensions in knitted cotton textiles.

CONCLUSIONS

Crude cellulases from *Trichoderma reesei* have been used to investigate the mechanisms of cellulase hydrolysis of cotton cellulose in the form of scoured and bleached fabric. Predictions of the general model for cellulase action were largely confirmed. An EG "rich" crude, from which CBH I and CBH II activity had been deleted showed typical endoglucanase activity, causing random chain scission which led to a rapid rise in fluidity and high strength loss but did not solubilize cotton. A crude, from which EG I and EG II activity had been deleted, caused less random hydrolysis (i. e. increased exo type activity) than the total crude mixture. Nevertheless, it solubilized cotton as effectively as the total crude mixture. This C-EGs did however retain some endo type activity which was possibly due to the endo character of both CBH I and II and also due to the undeleted EG III and V.

Since it is important to maintain fibre strength in most textile applications it does not appear to be appropriate to increase the endo activity of cellulases intended for cotton processing. There may however be advantages for low endoglucanase or endoglucanase free mixtures especially where substantial weight loss may be required to achieve softness or de-pilling without serious loss of strength.

The short treatments, as used in textile applications, keep the changes at the fibre surface while the long ones affect the internal structure of the fibre.

ESEM studies have revealed that the fibre retraction and twisting behavior of untreated fibres is absent after long treatments.

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25 Characterisation of paperboard designed for liquid containment

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Introduction

Many types and forms of polymers are used extensively in today's pac
need for extended self life along with special packaging criteria, means
required has to change to meet these needs. This is clearly evident in th
products. Liquid producers require packages that are cheap, strong, aes
and environmentally sound. By using virgin and recycled paperboard
advanced multi-layer polymer coatings, these criteria can be met by the
The main polymers that have been used to fulfil these requirements are:

- Poly(propylene)
- Poly(ethylene)
- Ethylene-vinyl alcohol copolymer

These polymers have been adapted, using new coating technology, to
of coatings that give the barrier properties that enable many types of liqu

These coating formulations are now established for the packaging
'aggressive' liquid products. However, the need to pack more 'chemically
growing and so new coating technologies will need to be develope
industries.

This paper shows a range of results from various techniques that
characterise three packaging systems. Characterisation of these produc
for future advancement.

The results will show why and how more 'chemically aggressive liquid