Dry action of *Trichoderma reesei* cellulases on cotton fabrics

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Following treatment with *Trichoderma reesei* cellulases, the mechanical properties of cotton fabrics were measured to assess the effect of adsorbed enzymes. The ability of adsorbed cellulases to act as anchors for further wet finishing processes was studied. Dried fabric samples, after 105 days under usual storage conditions, showed neither significant strength loss, nor any changes in the degree of polymerisation. After five months of storage, the enzymes were still found to be active. The adsorption of *T reesei* cellulases produced an increase in staining levels after dyeing with an acid dye, but washing fastness was poor. Washing with soap under alkaline conditions removed proteins almost completely. Adsorption of *T reesei* cellulases is reversible and desorption increases from pH 5 to pH 10. Because of the protein desorption under usual alkaline washing conditions, no practical application of adsorbed cellulases for further finishing processes is suggested and more work is required.

INTRODUCTION

Cellulases are well established in the finishing of cotton textiles. The best known applications are the depilling of cotton fabrics and the ageing of denim garments, where they are used to substitute, partly or completely, for pumice stones [1,2]. Cellulases have two different structural domains: a catalytic domain and a cellulose-binding domain (CBD) [3–6]. Cellulases of *Trichoderma reesei* are known to have their maximal activity at 50 °C and pH 5 in aqueous solution, but no information is available about their behaviour on dry fabrics.

Following treatment, the enzymes are usually inactivated and removed by washing with alkali or detergents. Otherwise the cellulases would remain adsorbed on the fabric and might retain their activity on the dry cotton under normal storage conditions. If cellulases could be immobilised on cotton fabrics, making use of their natural binding ability from the cellulose binding domains or by applying further fixation methods, they may serve as anchors for subsequent finishing procedures, such as dyeing. The introduction of amino acids onto the cotton surface enables the use of protein-specific dyes and finishing chemicals. Some advantages might be the use of more environmentally friendly dyeing and finishing methods, with the application of less salt and the use of less alkaline conditions. For any of these possible future applications, it remains to be established whether enzymes stay adsorbed or bound to the fabrics and do not attack fabrics during normal storing and washing conditions as,

otherwise, further treatments would have to be considered. At the moment no such information is available in the literature.

Three different enzyme compositions of *T reesei* cellulases were adsorbed on a mercerised cotton fabric. A study was undertaken on the effects of long-term storage and enzyme composition on the mechanical properties, degree of polymerisation of the fabrics and dyeability of cellulase treated fabrics with an acid dye that is commonly used in biochemistry for protein staining. Furthermore, the desorption at different pH values of the cellulases from the fabrics and the enzyme activities after storage were also investigated.

EXPERIMENTAL

Preparation of the cellulase treated fabrics

Woven fabric

Six pieces of mercerised cotton fabric (150 g), pre-washed with water for 20 min at 50 °C, were individually incubated for 20 min at ambient temperature (20–25 °C) in 10 l of a buffered solution (acetate buffer, pH 4.8–4.9) of *T reesei* cellulase (Röhm Enzyme, Finland) in a domestic washing machine. After the incubation, each fabric was wrung out, centrifuged and dried on a line. The solution wrung from the fabric was returned to the washing machine and the next fabric was introduced. For each cellulase we obtained six fabrics with decreasing amounts of adsorbed enzyme. Before and after introducing a fabric piece, the protein concentration in solution was determined by the method of Bradford [7].

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pared: total crude (TC = complete enzyme cocktail of *T reesei*), CBH-rich (cellobiohydrolase enriched product of a genetically-engineered strain of *T reesei* with deletions of major endoglucanase activities), EG-rich (enzyme cocktail rich in endoglucanases of a genetically engineered strain of *T reesei* with deletions of all cellobiohydrolase activities) [8].

The control fabric was just washed with buffer. Protein adsorbed onto the fabric was measured with modified Lowry method [9]. Values varied between 3 and 9 mg enzyme per g fabric [9]. Analyses were carried out in triplicate and samples were taken at three different points of the fabric.

Knitted fabric

Pieces consisting of 95 g of knitted cotton fabric were treated with enzymes TC, EG-rich and CBH-rich by a pad-batch method on a laboratory pad mangle (80% wet pick-up, 10 mg enzyme/g fabric, pH 4.8–4.9, acetic acid/sodium hydroxide buffer). After 30 min of incubation at ambient temperature, the fabric pieces were washed with water in a domestic washing machine to remove excess protein and were then dried on a line. Final protein concentrations on the fabric pieces, measured with a modified Lowry method [9], were 2.5 mg/g for TC cellulase, 2.3 mg/g for EG-rich and 2.6 mg/g for CBH-rich.

The dried fabrics were stored under controlled conditions (temperature = 21 °C, RH = 70–80%). To stop enzyme activity and to wash-off proteins; samples of the fabric were washed after 4, 17, 48 and 105 days in 5% sodium carbonate at 50–70 °C for 20 min.

Dyeing, washing and desorption tests

Fabric samples of 1g were dyed in a 25 ml aqueous solution (liquor ratio 25:1, pH 5.8) of Coomassie Brilliant Blue 250G (Merck, CI Acid Blue 90), 2 g dye/100 g fabric, in a Roaches dyeing machine (25 min⁻¹). Dyeing was started at 30 °C; the temperature was raised to 60 °C at a rate of 1 degC/min and was held there for 60 min. The fabric samples were then rinsed with cold water and dried.

Dye fastness was tested according to a variation of standard procedure ISO 105-C03:1978. Washing tests were carried out in an Rotawash machine (40 min⁻¹) at 60 °C for 30 min with either distilled water or an aqueous solution of 5 g/l neutral standard soap plus 2 g/l sodium carbonate. Reflectance (*K/S*) was measured before and after washing with an ACS Chroma Color spectrometer at 600 nm at three to five different points of the sample.

For desorption tests, 1 g of cellulase treated fabric was washed in a Rotawash machine (40 min⁻¹, 1 h at 30 °C and another hour at 50 °C) with 50 ml of aqueous buffer solution (pH 5: 0.1 M acetic acid/sodium hydroxide, pH 7: 0.01 M potassium dihydrogen orthophosphate/sodium hydroxide, and pH 10: 0.005 M sodium bicarbonate/0.01 M sodium hydroxide).

Protein in solution was measured by the Bradford method [7], and reducing soluble sugars were analysed as described above [9].

Comparative measurements of the resistance of the woven fabrics were made in warp direction with an Instron 1122 machine, applying a maximum force of 500–1000 N and with a minimum of seven trials per fabric.

Resistance of the knitted fabric was measured by the psi-burst test with at least five measurements per fabric. Strength loss was calculated as follows:

$$\text{Strength loss (\%)} = \frac{R_r - R_s}{R_r} \times 100 \quad (1)$$

where R_r is the reference resistance and R_s is the resistance of the sample.

Determination of the molecular weight distribution of cellulose

The molecular weight distribution of both the original and the enzyme-treated fabrics was obtained by gel permeation chromatography (GPC) of their tricarbanyl derivatives [10,11]. After milling to pass a 1 mm screen, 50 mg of each sample were transferred to a screw-cap assay tube and dried overnight with phosphorus pentoxide. To the dried material, 3 ml anhydrous pyridine and 0.5 ml phenyl isocyanate were added. The reaction was carried out for 48 h in a heating block previously set at 80 °C [0]. The reaction was stopped by adding 2 ml methanol and the final mixture was precipitated in an excess of a methanol/water solution (4:1). The precipitate was washed thoroughly with methanol and water until free of pyridine, then dried overnight with phosphorus pentoxide. The pyridine-free cellulose derivative was finally dissolved in tetrahydrofuran (THF) to give concentrations of about 0.5 mg/ml for GPC analysis. Alternatively, the cellulose tricarbanylate was recovered after evaporation of the reaction solvents. Co-evaporation with toluene was performed to eliminate residual pyridine. Both isolation strategies resulted in identical GPC profiles.

GPC of the tricarbanyl derivatives was carried out on a Shimadzu LC10AD liquid chromatograph. Samples of cellulose tricarbanylate in THF were filtered through a Teflon membrane with a pore size of 0.45 µm and analysed at 45 °C using a series of one guard column and four Tosoh TSK-GEL columns (7.8 × 300 mm) with exclusion limits of 4×10^7 (TSK 6000 HXL), 4×10^5 (TSK 4000 HXL), 6×10^4 (TSK 3000 XHL) and 1×10^3 (TSK 1000 HXL) units of molecular weight [12]. THF was used as the eluting solvent at a flow rate of 1 ml/min. A minimum of three analyses was carried out for each replicate. The samples in the eluent were monitored by UV absorbance using a Shimadzu SPD-10A UV detector at the wavelength of 254 nm. The GPC data was treated using the GPC utility of the Shimadzu LC workstation.

The GPC calibration curve was generated from the elution profile of 20 polystyrene standards with narrow

molecular weight distributions [13–15]. The universal parameters used for calibration (Mark-Houwink coefficients) were those reported by Valtasaari and Saarela [14], $K_p = 1.18 \times 10^{-4}$ and $\alpha_p = 0.74$ for polystyrene in THF, and $K_c = 2.01 \times 10^{-5}$ and $\alpha_c = 0.92$ for cellulose tricarbanilate in THF. The degree of polymerisation (DP) of cellulose was obtained by dividing the molecular weight of the tricarbanilated polymer by the corresponding molecular weight of the tricarbanil derivative of anhydroglucose (519). Both the number average DP and the weight average DP of the substrates were calculated as described previously [16].

RESULTS AND DISCUSSION

Dyeing and washing fastness of cellulase treated fabrics

The adsorption of fungal cellulase enzymes from *T reesei* onto cotton fabrics leads to an increase in colour yield after dyeing with the acid dye Coomassie Brilliant Blue G250 (Figure 1). No relationship between the extent of bound protein and colour yield was observed for any of the enzymes studied. The colour fastness of the protein-treated, dyed fabrics was very poor with more than 50% reduction of *K/S* value after washing in water and almost 80% after washing in neutral soap. All three cellulase mixtures (total crude, EG-rich and CBH-rich) showed similar behaviour (Figure 1).

Figure 1 Staining levels (at 600 nm) of dyed cotton fabrics treated with cellulases of *T reesei* before and after washing with water or soap

The increased affinity of the treated cotton fabrics for acid dye may be explained by the increased level of ionic groups on the fabric introduced by protein adsorption. Contrary to the results obtained with cellulases having a CBD of family II [17], no relationship between bound protein and colour yield was observed with different amounts of enzyme. This is probably due to the reversibility of the adsorption of *T reesei* enzymes, whose CBDs belong to family I [19]. Another or additional reason might be the high amount of protein on the fabric. A

probably reached since, at lower concentrations of up to 2 mg protein/g fabric, a more linear relationship between staining levels and bound protein was observed. We propose that at protein contents above 3 mg/g fabric the bound protein interferes with the dye–cellulose interactions and that part of the bound protein desorbs during dyeing.

Dyeing of protein-treated fabrics with Coomassie Brilliant Blue was found to be a good indicator for the evenness of protein adsorption for the fabric.

Dyeing of fabrics treated with bovine serum albumin fraction V (BSA), which does not contain a cellulose binding domain and, thus, does not have a special affinity to cellulose, resulted in unevenly dyed fabrics. In contrast, dye levelling of the cellulase-treated fabrics was much better.

Alkaline washing (50 g/l sodium carbonate) of fabrics in a washing machine before dyeing removed proteins completely and subsequent staining with Coomassie Brilliant Blue was lower than for untreated fabrics.

The poor washing fastness of the protein-treated and dyed fabrics to soap, and even to water, is due to the reversibility of the adsorption of the cellulose binding domains of the *T reesei* cellulases [19]. The CBDs of all known *T reesei* cellulases belong to family I and are short peptides of about 30 amino acids. This may also explain the similar behaviour of the three different cellulase mixtures. The binding of cellulases to cellulose is mainly because of the relatively weak hydrophobic interactions of their cellulose binding domains. As recently reported [18], a significantly higher staining of cotton with acid dyes may be obtained with thermofixation of the proteins by ironing of the fabric before dyeing. Due to the thermal denaturation of the adsorbed proteins additional hydrophobic protein–cellulose interactions are formed. However, washing fastness could not be improved sufficiently. Considering also the recently reported partial desorption of CBDs of family II under alkaline washing conditions [17], this means that simple adsorption of cellulase proteins to cotton fabrics is not sufficient to fix acid dyes to cotton with a reasonable washing fastness.

Desorption of *T reesei* cellulases

The desorption of cellulases was tested in the pH range of 5–10, and it was shown that desorption increases almost linearly with rising pH. Even at pH 5 more than 15% of the initially adsorbed TC protein was desorbed at 30 °C (Figure 2). No significant difference was found between desorption at 30 °C and 50 °C. No differences were found in the behaviour of EG-rich and CBH-rich. For the desorption of *T reesei* cellulases from a cotton fabric a change in pH seems to have a greater effect than a change in temperature.

Even after more than five months, the desorbed cellulases were still found to be active (Figure 2). This means that, for the possible application of adsorbed cellulases, enzymes have to be deactivated, otherwise they

Figure 2 Desorption and activity of TC cellulases of *T reesei* after absorption onto cotton fabric for longer than five months

Figure 3 Effect of adsorbed cellulases of *T reesei* on warp strength loss of a woven cotton fabric during long term storage (20–25 °C, RH 65–75%)

Figure 4 Strength loss of a knitted fabric adsorbed with *T reesei* cellulases during long-term storage under standard conditions

conditions. As recently reported, wet ironing of the fabrics inactivates cellulases completely, whereas dry ironing resulted in only partial deactivation of the enzymes [18]. The same was found for CBH-rich and EG-rich enzymes.

Effect of adsorbed cellulases on strength loss and DP

No significant strength loss and no differences between adsorbed enzymes were found after 105 days of storage of

Similar results were found for knitted fabrics treated with cellulases stored over 380 days (Figure 4). The initial strength loss already observed in the beginning of storage and the differences between the enzymes, which coincided with activities of these enzymes on cotton reported by Cavaco-Paulo *et al.* [8], may be ascribed to cellulase attack during the adsorption step. These results were confirmed by the measurements of the DP of the woven fabrics where no differences between treated and untreated fabrics were found (Table 1).

Table 1 Degree of polymerisation and polydispersity of cellulase treated woven fabrics (> 150 days)

Adsorbed cellulases	DP	Polydispersity
Control	3188 ± 155	3.48 ± 0.33
Total crude	3075 ± 111	3.13 ± 0.06
EG-rich	3181 ± 79	3.26 ± 0.36
CBH-rich	3198 ± 95	3.89 ± 0.24

This means that, even after long-term storage of cotton fabrics treated with active cellulases under standard conditions, no noticeable degradation nor destruction of the fabrics is to be expected. The most important factor in these results is probably the low amount of water present in the storage conditions. Even in their active conformation, the adsorbed enzymes are not able to act significantly on the cotton cellulose. Owing to the lack of water, the enzymes probably remain virtually inflexible and immobile in their bound state. This finding may be compared to work on enzyme activity in organic solvents, where a critical thermodynamic water activity was defined [20]. Furthermore, because of the absence of reaction media (e.g. water) in the dried state, the transport of substrates and products cannot occur to any great extent.

CONCLUSIONS

The adsorption of *Trichoderma reesei* cellulases onto a cotton fabric increases its dyeability with acid dyes, probably owing to an increase in ionic groups on the fabric's surface. Because of the reversibility of cellulase adsorption, washing fastness, especially under alkaline conditions, is very poor. The desorption of cellulases increases from pH 5 to 10.

Even after long-term storage of a cotton fabric treated with *T reesei* cellulases, no noticeable degradation, no change in the degree of polymerisation and no significant strength loss were observed. This may be explained by the absence of water as a reaction medium, which is essential for adsorption and desorption of enzymes, as well as for the transport of substrates and products. The cellulases remained active even after longer than five months, staying adsorbed on the cotton fabrics under dry

conditions. Thus, for a possible application, such cellulases should be inactivated by a prior genetic modification of the catalytic site, or by thermal denaturation. Otherwise, unaltered cellulases may attack cellulosic fibres when fabrics are wetted.

However, from the results obtained, no direct application of the use of the cellulases as anchors for final finishing processes can be suggested. To confer better resistance against usual alkaline washing processes, more work needs to be undertaken on fixing the cellulases. This could possibly be achieved by additional chemical binding.

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REFERENCES

1. A Cavaco-Paulo, *Processing textile fibres with enzymes*, Ed. K F Eriksson and A Cavaco-Paulo, ACS Symp. Series, **687** (1998) 180.
2. K Maurer, *Development of new cellulases*, Ed. M Dekker, J H van Ee, O Misset and E J Baas, Surfactant Science Series, **69** (1997) 175.
3. B Henrissat, *Biochem. J.*, **280** (1991) 309.
4. B Henrissat and A Bairoch, *Biochem. J.*, **293** (1993) 781.
5. B Henrissat and A Bairoch, *Biochem. J.*, **316** (1996) 695.
6. G Davies and B Henrissat, *Structure*, **3** (1995) 853.
7. M Bradford, *Anal. Biochem.*, **72** (1976) 248.
8. A Cavaco-Paulo, L Almeida and D Bishop, *Text. Res. J.*, **68** (1998) 273.
9. A Cavaco-Paulo, J Morgado, L Almeida and D Kilburn, *Text. Res. J.*, **68** (1998) 398.
10. L R Schroeder and F C Haigh, *Tappi*, **62** (1979) 103.
11. I Kossler, J Danhelka and M Netopilik, *Svensk. Papperstidn. Res.*, **18** (1981) 137.
12. L P Ramos, M M Nazhad and J N Saddler, *Enzyme Microb. Technol.*, **15** (1993) 821.
13. H Coll, and D K Gilding, *J. Polymer Sci. A-2*, **8** (1970) 89.
14. L Valtasaari and K Saarela, *Paperi ja Puu*, **57** (1975) 5.
15. J Danhelka and I Kossler, *J. Polymer Sci.*, **14** (1976) 287.
16. W W Yau, J J Kirkland and D D Bly, *Modern size exclusion liquid chromatography* (New York: John Wiley & Sons, 1979) 7.
17. A Cavaco-Paulo, J Morgado, J Andreaus and D Kilburn, *Enzyme Microbiol. Technol.*, **25** (1999) 639.
18. J Andreaus, H Azevedo and A Cavaco-Paulo, *J. Mol. Cat. B*, **7** (1999) 233.
19. M Linder, T Teeri, *Proc. Nat. Acad. Sci.*, **93** (1996) 12251.
20. A M P Koskinen and A M Klibanov, *Enzymatic reactions in organic media* (Glasgow: Blackie, 1996) 14, 49.