EXPLORING ENZYMES ON COTTON AND THEIR PRODUCT TARGETS

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Abstract

Enzyme-active cotton is a functional biocompatible material, and has potential applications as a sustainable material. With this in mind we have explored development of enzyme-active cotton with product potential as a disposable or reusable textile material. Lysozyme, which historically has been a well studied enzyme, was employed as a model enzyme since it has broad antimicrobial activity with antibacterial, antiviral, and antifungal applications. Lysozyme was covalently attached to three cotton fabrics using both an aqueous and organic based linking strategy. The antibacterial activity and several fiber properties that are signature characteristics of the enzyme-cellulose conjugate including zeta potential, and infra-red spectra were elucidated. Organic solvent-based carbodiimide coupling gave higher incorporation when compared with an aqueous based approach. The organic solvent approach with cotton twill using a carbodiimide-mediated coupling reaction gave the highest incorporation of lysozyme (16.1milligrams/gram cotton). Cotton spunlaced nonwoven gave slightly lower incorporation of lysozyme (12.8 milligrams/gram cotton) with highest antimicrobial activity, and woven print cloth yielded less (4 milligrams/gram cotton). The effect of storage on activity was monitored over a five month period, and as much as 60-70 per cent of enzyme activity remained after five months of storage.

Introduction

Cotton is an environmentally friendly and sustainable material that is an attractive option as a biocompatiable textile surface for clothes and biomedical materials. Recent interest in the development of antimicrobials attached to cotton materials includes modifications that function through fiber entrapment with slow release activity, and covalent modifications designed to inhibit bacterial growth directly on the fiber. Several approaches to incorporating silver, chitosan, and regenerable organic functionalities including halamine and iodine have been recently reported (Parikh et al.; Tomsic et al.; Gupta et al.; Wedmore; Hirano; Lou; Nasr et al.; Chung et al.; Kim et al.; Gupta and Saini; Liu and Sun). C-type lysozyme is a 1,4-□-N-acetylmuramidase that derives its antibacterial activity from the selective lysis of the cell wall peptidoglycan at the glycosidic bond between the C-1 atom of N-acetylmuramic acid (NAM) and O-4 of N-acetylglucosamine (NAG) (Salton and Ghuysen, 1959 and 1960; Jeanloz et al.). The mechanism of action of lysozyme has been shown to occur through binding of tri-NAG in a cleft at the surface of the enzyme and subsequent carbonium ion – mediated hydrolysis of the glycosidic bond between the NAM and NAG residues (Philips; Blake et al, 1967a,b). The mechanism of action of lysozyme is probably the best studied of any enzyme reported to date. Despite lysozyme's antibacterial (Salton and Ghuysen 1960) and antiviral activity (Cisani et al.; Samaranayake et al.; Lee-Huang et al.) little has been reported on developing a cotton-based antimicrobial fabric using this enzyme. With this in mind we have explored several approaches to attaching lysozyme to different cotton fabrics while retaining enzyme activity over with longevity assessment.

Esterification of cotton cellulose with 9-fluorenylmethyloxycarbonyl (Fmoc) - glycine

Desized, scoured, bleached and mercerized cotton twill, print cloth and nonwoven fabric were used for the synthesis. Cotton cellulose fabric was esterified through base-catalyzed carbodiimide/ hydroxybenzotriazole (HOBT) acylation. Cotton samples were pre-treated with 25% trifluoroacetic acid in methylene chloride (DCM) for 10 minutes, washed with DCM, 10% diisopropylmethylamine in DCM and washed with DCM. Then, cotton samples were dried in a Buchner funnel. The cotton samples were then placed in a beaker containing Fmoc-glycine (4.46g, 0.015mol), diisopropylcarbodiimide (DIC) (1.89g, 0.015mol), hydroxybenzotriazole (HOBT) (2.03g, 0.15mol) and dimethylaminopyridine (DMAP) (0.18g, 1.5mmol) in 50mL of dimethylformamide (DMF). This beaker was placed in an ultrasonic bath for 90 minutes. Deprotection of the Fmoc-glycine was accomplished in 20% piperidine/dimethylformamide for 15 minutes and washed with DMF and DCM.

Immobilization of lysozyme on glycine-cotton with DIC

To a beaker were added lysozyme (3.74g, 0.25mmol), DIC (0.315g, 2.5mmol) and HOBT (0.338g, 2.5mmol) in 50mL of DMF. The cotton samples were added to the solution and the beaker was placed in the ultrasonic bath for 3h at room temperature. The samples were washed with DMF, ethanol and then DCM.

Immobilization of lysozyme on glycine-cotton with carbonyldiimidazole (CDI)

Lysozyme (0.565g, 3.8E-05mol) and CDI (1.70g, 0.010mol) were allowed to react in 60mL of DMF for 2 h. Glycine-cotton, 6.8g pre-swollen in DMF, was added to the lysozyme/CDI solution and allowed to react at 4°C overnight. The samples were washed extensively with 0.1mM phosphate buffer.

Immobilization Using Citric Acid Treated Cotton Fabric

The CA-cotton fabrics were placed in a 0.2M sodium phosphate solution at pH 4.75 containing 0.529g, 1.25mmol, of CMCS. The samples were shaken for 30 minutes, filtered and washed with neutral phosphate buffer. The activated fabrics were then added to a phosphate buffered lysozyme solution with a concentration of 10mg/mL at pH7. The samples are placed in the fridge at ~4-8°C for 19hrs. The filtrate and washings were collected for analysis to estimate protein content.

Immobilization Using Aminosilanated Treated Cotton Fabric

The cotton fabric was treated with 5%(wt.) solution 3-aminopropyltriethoxysilane (APTES) hydrolyzed in a 3:1 ethanol:water. Lysozyme was dissolved in a sodium phosphate solution, pH 4.5 with a concentration of 10mg/mL. The WS carbodiimide, 0.144g or 0.34mmol, was added and the mixture stirred for 45 minutes. The AS-cotton fabrics were placed in the lysozyme/CMCS solution, the pH adjusted to 7 and placed in the fridge at 4-8°C for 19hrs. The filtrate and washings were collected for analysis.

Antibacterial Assay

The antibacterial activity was measured based on the lysis of *Micrococcus lysodeikticus* cell, monitoring the decrease in optical density at 450nm. The assays were done using 48-well culture plates and a benchmark plate reader (Bio-tek) at 25 °C. The cotton samples were pulverized on a Wiley Mill of 20 mesh screen prior to assaying the fibers for antibacterial activity. Fifty milligrams of the lysozyme linked fibers were suspended in 5mL of 66mM phosphate buffer, pH 6.24, for 1-2 h period by shaking. One-hundred microliters of the above fiber suspension was combined with 400□L of buffer totaling 500□L. For comparison, a lysozyme standard curve was conducted simultaneously using a stock solution of 950 Units/mL in phosphate buffer. To each sample and control well 500□L of 0.05% (w/v) suspension of *M. lysodeikticus* in 66mM phosphate buffer were added. Following this addition to the plate wells, the progress of the experiment was monitored at 450 nm at 25°C for 6 h.

Results and Discussion

Cotton is an environmentally friendly and sustainable material and has recently been combined with bio-based antimicrobial finishes. Hydrolase enzymes that confer antimicrobial activity also provide a potential barrier to microbial invasion through hydrolysis of cell wall polysaccharides. Cotton-linked hydrolases, which target degradation of the bacterial cell wall, have shown promise as active immobilized enzymes with antimicrobial activity on cotton (Edwards et al., 2000; Ibrahim et al.). The potential utility of immobilized enzymes on cotton fabrics as an alternative to other antimicrobial textile or nonwoven surfaces is that it offers a highly selective and environmentally acceptable material. Thus, cotton cellulose needs to be further explored for its variety of fabric substrate properties to immobilize enzymes with antimicrobial and decontamination activity for use in medical hygienic applications (Edwards and Goheen).

This paper compares linking strategies using both an organic and aqueous phase coupling approach. The level of lysozyme incorporation is shown in Tables 1 and 2 for each type of fabric construction and the synthetic approach used. Coupling efficiency of the enzyme to the fabric was determined by analysis of percent nitrogen and proteinaceous amino acid incorporated into the cellulose fibers of the fabrics. As shown in Table 1 lysozyme attachment to cotton twill, nonwoven and print cloth fabric was 16, 12, and 4 milligrams per gram of cotton respectively. In comparison in Table 2 the aqueous coupling routes to the enzyme attachment on cotton twill, nonwoven and printcloth were considerably less. A comparison of IR spectra taken of the lysozyme-cellulose conjugate on treated cotton nonwoven with untreated cotton nonwoven is shown in Fig. 1. The IR spectra show protein levels and absorbance ratios greater for the treated versus the untreated fabric. The absorbance at 1650 cm^{-1}

and 1515 cm^{-1} undergo an increase as lysozyme is bound to the cotton. These increases are due to the amide I and amide II frequencies at 1650 cm^{-1} and 1515 cm^{-1} respectively, which are characteristic of proteins.

Potential Product Considerations

Combining a low cost process and the lower cost commercially available enzymes should make it attractive to industry to produce antimicrobial and decontamination wipes based on nonwoven cotton. For example typical protease costs are around 11-13 USD/kg. This number can vary based on the specific enzyme, size of a customer's account, competitive pressures, production and packaging, as well as other special agreements on pricing. Using the results from our studies, an estimated cost of $0.005/g fabric was calculated for a commercially available protease.

Aging Study

The results of a five month aging study are shown in Figure 2. As seen approximately 40 percent of the activity was lost between two and five months after the initial preparation. Thus, more work needs to be done to address retention of activity during storage. For example the incorporations of phorbol esters, or hydrated polysaccharides into the enzyme and fabric may act as preservatives and improve the longevity of the fabric.

Table 1. Levels of lysozyme conjugated to cotton fabric using a conjugation method as outlined in the Materials and Methods which employs dicyclohexylcarbodiimide (DIC) and an organic solvent for the reaction.

Sample Description	Nitrogen%	Protein/Fabric (mg/g)
control cotton twill	0.19	
gly-cotton twill	0.23	
lysozyme-**DIC**-gly-cotton twill	1.84	12
control -non woven	0.19	
gly-non woven	0.21	
lysozyme-**DIC**-gly-non woven	1.49	16
control print cloth	0.15	
gly-print cloth	0.23	
lysozyme-**DIC**-gly-print cloth	0.63	4

Table 2. Lysozyme incorporation on cotton fabrics; Abbreviations: UT = untreated, CA = citric acid crosslinked, PC = print cloth, NW = Nonwoven, TW = twill, AS = aminosilane.

Sample Description	N%	LYZ N%	Protein/Fabric (mg/g)
UT- Print cloth	0.183		
CA- PC	0.180		
LYZ-CA-PC	0.229	0.046	0.4600
UT-Nonwoven	0.153		
CA- NW	0.235		
LYZ-CA-NW	0.293	0.14	1.400
UT-TW	0.158		
AS-TW	0.259		
LY-AS-TW	0.226	0.0678	0.6784
UT-PC*	0.183		
AS-PC	0.250		
LY-AS-PC	0.232	0.0485	0.485
UT-NW*	0.153		
AS-NW	0.313		
LY-AS-NW	0.191	0.0379	0.3787

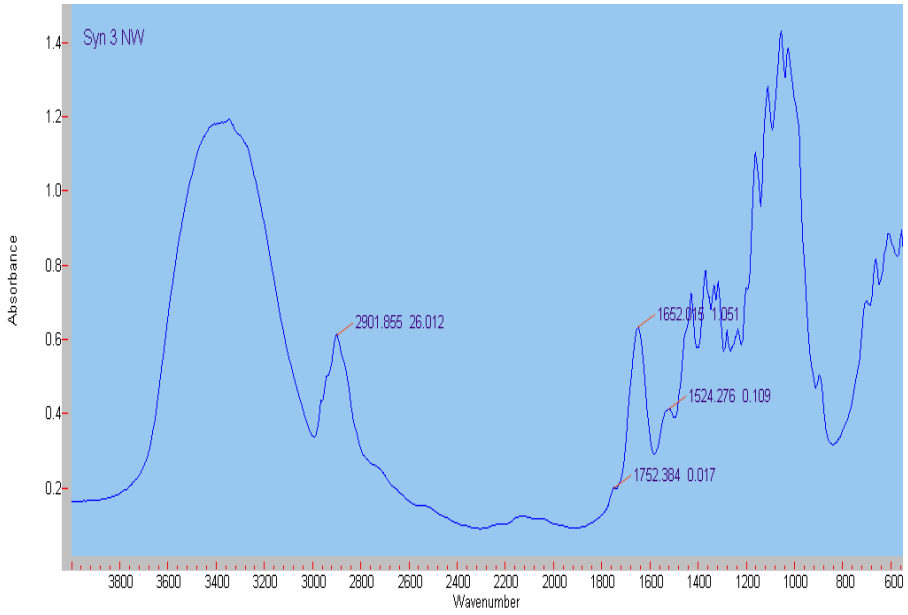

Figure 1: IR spectrum of lysozyme-conjugated cotton fabric. Amide I and Amide II stretching frequencies are indicated in red, and denote the presence of lysozyme in the fabric.

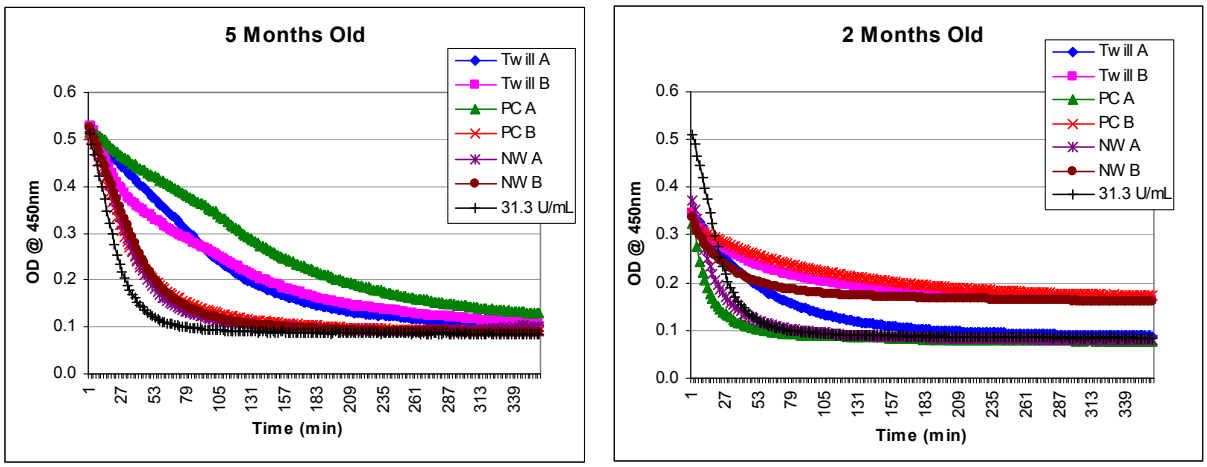

Figure 2: Aging study of lysozyme bound cotton fabrics. As seen after two month there is still significant activity present on all fabrics. The average percent active bound lysozyme ranges from an average of 50 – 100 percent after two months, and from10 – 60 per cent after five months.

Summary

This study of covalently attaching lysozyme to cellulose-cotton fabrics and measuring the activity of the resultant cotton enzyme conjugates demonstrated the efficacy on three different types of woven and nonwoven fabrics. More work is required to improve the product yield. Improved yields may be realized by improving the enzyme linker titer on the cotton fiber. Improved incorporation of carboxylate or amino functionality on the cotton fiber will improve the coupling synthesis yield, and it should improve activity. Activity is robust on the cotton fabric while the enzyme is active, and the enzyme-cellulose conjugate probably stabilizes the enzyme against proteolytic degradation.

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