

**IMMOBILIZATION OF LYSOZYME ON COTTON FABRICS; SYNTHESIS, CHARACTERIZATION, AND ACTIVITY****Nicolette Prevost****J. Vincent Edwards****Brian Condon****Sarah Batiste****Phyllis Howley****Kandan Sethumadavan****Jaffor Ullah****Southern Regional Research Center, USDA-ARS****New Orleans, LA****Abstract**

The antimicrobial activity of lysozyme derives from the hydrolysis of the bacterial cell wall polysaccharide at the glycosidic bond that links N-acetyl-glucosamine and N-acetyl-muramic acid. Maintaining the activity of lysozyme while bound to a cellulose substrate is a goal of enzyme immobilization on cotton and is essential to developing functional cotton-based decontamination wipes. Here we outline the covalent attachment of lysozyme to glycine derivatized cotton as a model for its potential use in antimicrobial cotton products. Lysozyme was immobilized on cotton printcloth, twill, and nonwovens using three separate routes to enzyme immobilization. The attachment of lysozyme to cotton fibers was made through a glycine amino acid linker esterified to the cotton cellulose. The paper will discuss the resulting activities of the cotton fabrics and the immobilization chemistries used to link the lysozyme.

**Introduction**

The highly absorbent, non-toxic, and soft-hand properties of cotton make it an attractive option as a biocompatible textile surface. The potential utility of immobilized enzymes on cotton fabrics as a source of biologically active textile surfaces offers highly selective functional properties and an environmentally acceptable finish. Thus, cotton cellulose may ideally act as a medium to immobilize enzymes with antimicrobial, decontamination, or specialty properties for use in medical, hygienic and decontamination applications.

A variety of preparative approaches including covalent binding, cross-linking and adsorption have been utilized to attach enzymes and other biologically active compounds to cotton fabrics (1). Previously in our laboratory, we have studied different bio-active cotton models for applications in wound healing bandages, cotton resurfacing and antimicrobial products. Enzyme-active fabrics have numerous potential uses. Some applications may include medical, antimicrobial and hygienic products for enzymes that digest and kill bacteria. Enzymes immobilized on fibers could also catalyze very specific reactions either for industrial processes (2), or for inactivation of specific chemical warfare agents (3). One concern in developing these products is the retention of enzyme activity following conjugation of the enzyme to the cotton fabric. In this paper we explore synthetic routes to covalently attach lysozyme to cotton efficiently while retaining biological activity.

**Materials and Methods****Esterification of cotton cellulose with F-moc glycine**

Desized, scoured, bleached and mercerized cotton twill, printcloth and nonwoven fabric were used for the synthesis. Esterification of cotton cellulose fabric was accomplished through base-catalyzed carbodiimide/HOBT acylation. Cotton samples were pre-treated with 25% trifluoroacetic acid in methylene chloride [DCM] (10min), 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 a 0.3M solution of F-moc glycine/diisopropylcarbodiimide/hydroxybenzotriazole and 0.03M of dimethylaminopyridine in dimethylformamide. This beaker was placed in an ultrasonic bath for 90 minutes. Deprotection of the f-moc glycine was accomplished in 20% piperidine/dimethylformamide for 15 minutes and washed.

**Immobilization of lysozyme on glycine-cotton with diisopropylcarbodiimide**

To a beaker was added 13mM solution of lysozyme and 0.05M diisopropylcarbodiimide/ hydroxybenzotriazole in

dimethylformamide. 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 dimethylformamide, ethanol and then methylene chloride.

#### **Immobilization of lysozyme on glycine-cotton with glutaraldehyde**

After soaking glycine-cotton samples for an hour in 0.1mM potassium phosphate buffer, samples were placed in a beaker containing 25% glutaraldehyde solution [5mL per 600mg of glycine-cotton] and then placed in a ultrasonic bath filled with ice for an hour. It was then refrigerated for 2 to 3 days. The samples were washed and placed in a solution of lysozyme [250mg per 600mg of gly-cotton] in phosphate buffer and returned to the ultrasonic bath for an hour. Samples were refrigerated for 2 to 3 days and washed with buffer solution.

#### **Immobilization of lysozyme on glycine-cotton with carbonyldiimidazole [CDI]**

A ratio of 1:3:12 of lysozyme: carbonyldiimidazole: gly-cotton was used. The lysozyme and CDI was allowed to react in dimethylformamide for 2 h. Pre-swollen gly-cotton 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.

#### **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 by shaking. One-hundred microliters of the above fiber suspension was combined with 400μL of buffer totaling 500 μL. For comparison, a lysozyme standard curve was conducted simultaneously using a stock solution of 950 U/mL in phosphate buffer. To each sample and control well 500 μL 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 450nm at 25°C for 6 h.

### **Results and Discussion**

#### **Immobilization of lysozyme on glycine-cellulose cotton**

Lysozyme was covalently attached to woven and nonwoven cotton fabrics using three synthetic routes based on coupling reactions that resulted in retention of biological activity. In the organic phase approaches, protein is directly linked to cotton by synthetic activation of the protein functional group or by synthesizing it directly on the cotton in a stepwise manner. The amino group of the glycine esterified cellulose is coupled to the carboxylate end of the side chains of the lysozyme using the coupling agents, diisopropylcarbodiimide [DIC] and carbonyldiimidazole [CDI]. In the aqueous phase coupling reaction, the functional group of the fabric is activated and is coupled to the protein. Glutaraldehyde [GA] is used to couple the amino group of the glycine bound fabric with the amino group (lysine) side chains of lysozyme. Figure 1 illustrates the proposed synthetic routes of conjugating lysozyme to glycine esterified cellulose.

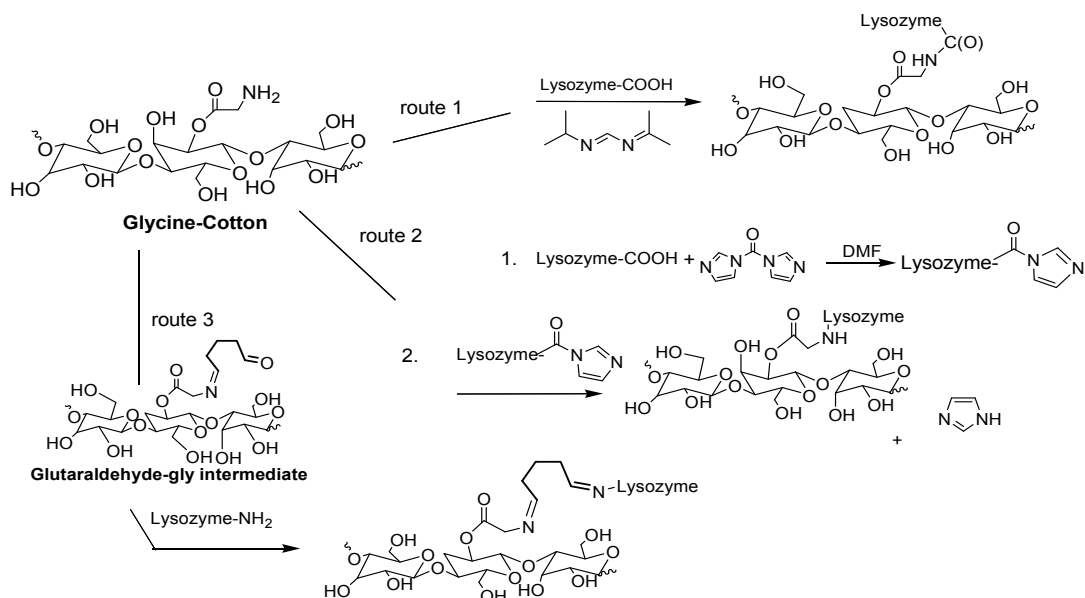


Figure 1. Synthetic routes to immobilized lysozyme on glycine cotton.

The protein levels for each type of fabric construction and technique are shown in Table 1. Coupling efficiency was determined by analysis of percent nitrogen and proteinaceous amino acid. With each type of fabric, glutaraldehyde coupling did not show an appreciable amount of lysozyme attached to the fabric according to the nitrogen analysis. This result was also supported by the low lysozyme activity evident in the assay. In the case of glutaraldehyde, this coupling agent acts as a “crosslinking” agent that is part of the final product. Because it is also flexible, it could potentially crosslink other glycine amino groups on the cotton or form cyclic diimine as side products which would hinder coupling with the amino group of the side chain of the protein.

Table 1. Levels of lysozyme conjugated to cotton fabric and relative antibacterial activity

Sample Description	Nitrogen%	mol/mg	%protein
control cotton twill	0.19		
gly-cotton twill	0.23		
lysozyme- <b>DIC</b> -gly-cotton twill	1.84	8.91E-06	12.78
lysozyme- <b>GA</b> -gly-cotton twill	0.06		
lysozyme- <b>CDI</b> -gly-cotton twill	0.84	4.32E-06	6.19
control -non woven	0.19		
gly-non woven	0.21		
lysozyme- <b>DIC</b> -gly-non woven	1.49	7.09E-06	10.16
lysozyme- <b>GA</b> -gly-non woven	0.15		
lysozyme- <b>CDI</b> -gly-non woven	0.97	4.54E-06	6.51
control printcloth	0.15		
gly-printcloth	0.23		
lysozyme- <b>DIC</b> -gly-printcloth	0.63	2.21E-06	3.17
lysozyme- <b>GA</b> -gly-printcloth	0.17		
lysozyme- <b>CDI</b> -gly-printcloth	0.90	3.71E-06	5.32

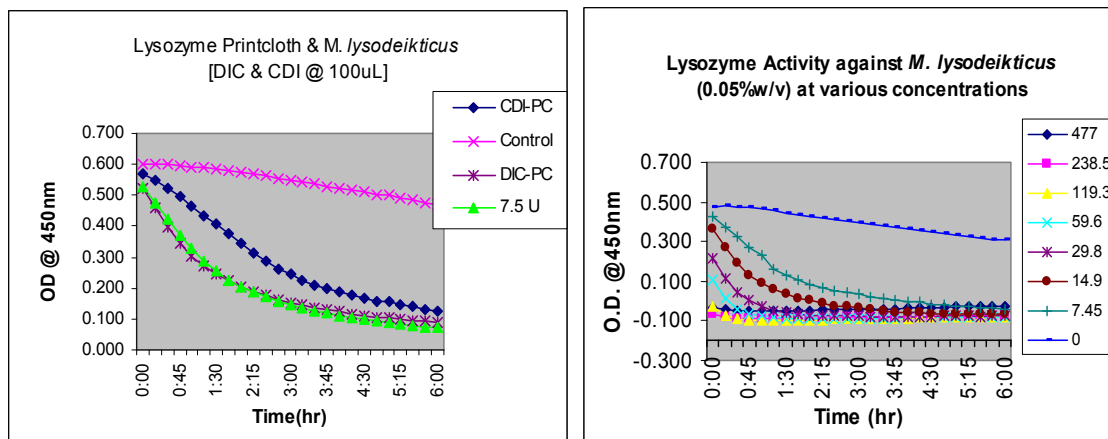


Figure 2. The left graph depicts the activity of lysozyme bound to print cloth by carbodiimide and carbonyldiimidazole routes against 0.05%(w/v) *M. lysodeikticus* over the course of 6 h. The experiment was carried out in triplicate and mean values were used to plot the graph. The graph on the right depicts the activity of lysozyme in solution at various concentrations against 0.05%(w/v) *M. lysodeikticus* over the course of 6 h.

### Enzymatic Activity

Lysozyme cleaves the bacterial cell wall polysaccharide at the glycosidic bond, inducing cell lysis. To evaluate the retained activity of the lysozyme bound cotton fabrics, cotton samples were pulverized and suspended in buffer and aliquots of this liquid were mixed with a suspension of *M. lysodeikticus*. Its liquid turbidity was monitored spectrophotometrically every 5 minutes for 6 h. Figure 2 shows the activity of the lysozyme bound print cloth using coupling agents **DIC** and **CDI** against the suspension of bacteria cells for 6 h. The untreated control was also pulverized and suspended in buffer, mixed with a suspension of the bacteria, and monitored in parallel. The control mimics the bacteria alone in buffer and shows the same autolysis pattern. The activity of the lysozyme bound printcloth sample, using carbodiimide coupling method, measures ~ 7.5 U against the standard dilution. The specific activity of lysozyme in solution was 47,700 Units/mg; the immobilized activity was equivalent to 157ng of enzyme in solution.

### Summary

This study reviewed methods that covalently attached lysozyme to cotton fabric and retained biological activity. Here the organic phase coupling procedure yielded higher immobilized lysozyme on glycine cotton fabric. The enzyme that was bound to fabric maintained activity after storage. Further investigation is warranted to identify more efficacious linking chemistries and sample evaluation.

### References

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