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COTTON STUDY: ALBUMIN BINDING AND ITS EFFECT ON ELASTASE ACTIVITY IN THE CHRONIC NON-HEALING WOUND

NATHAN CASTRO, STEVE GOHEEN

ABSTRACT

Cotton, as it is used in wound dressings is composed of nearly pure cellulose. During the wound-healing process, cotton is exposed to various blood components including water, salts, cells, and blood proteins. Albumin is the most prominent protein in blood. Elastase is an enzyme secreted by white blood cells and takes an active role in tissue reconstruction. In the chronic non-healing wound, elastase is often over-expressed such that this enzyme digests tissue and growth factors, and interferes with the normal healing process. Our goal is to design a cotton wound dressing that will sequester elastase or assist in reducing elastase activity in the presence of other blood proteins such as albumin. The ability of cotton and various cotton derivatives to sequester elastase and albumin has been studied by examining the adsorption of these two proteins separately. We undertook the present work to confirm the binding of albumin to cotton and to quantify the activity of elastase in the presence of various derivatives of cotton. We previously observed a slight increase in elastase activity when exposed to cotton. We also observed a continuous accumulation of albumin on cotton using high-performance liquid chromatography methods. In the present study, we used an open-column-absorption technique coupled with a colorimetric protein assay to confirm losses of albumin to cotton. We have also confirmed increased elastase activity after exposure to cotton. The results are discussed in relation to the porosity of cotton and the use of cotton for treating chronic non-healing wounds.

INTRODUCTION

Decreasing the negative effects of chronic non-healing wounds on a patient's comfort level, improving the economic relief, and exploring potential commercial benefits are all justifiable bases for continuing the research efforts to develop treated cotton gauze that will help expedite the wound-healing process. Although many individuals suffer from non-healing wounds, those who are diabetic are especially susceptible to such wounds which, in some cases, lead to the amputation of the affected limb. More than 60% of non-traumatic lower-limb amputations occur among people with diabetes. In 2000-2001 that percentage corresponded to approximately 82,000 cases. Medical costs related to amputation can exceed $13,000 per patient and the overall indirect medical costs, including loss in productivity, are as high as $39.8 billion per year [1].

Another segment of the U.S. population, some 200,000 people, lives with a disability related to spinal cord injuries. This segment's medical care costs the nation approximately $9.7 billion per year. Of that amount, $1.2 billion is attributed to the secondary condition of pressure sores [2]. The costs and care of pressure ulcers is so significant that advances in chronic wound research and fiber dressing design for accelerated healing is clearly needed.

During the normal healing process, elastase and other proteases help digest unwanted proteins, thereby removing them from the wounded region. Wound fluid from chronic non-healing wounds

Solution	Buffer (mL)	BSA (mL)	BSA (µg)
1	0.5	0	0
2	0.4	0.1	60
3	0.3	0.2	120
4	0.2	0.3	180
5	0.1	0.4	240
6	0	0.5	300

Table 1. Volume of solutions used in constructing BSA standard curve.

Elastase (µg/mL)	Elastase (mL)	Saline Solution (mL)
0	0	0.5
1.14E+02	0.1	0.4
2.28E+02	0.2	0.3
3.42E+02	0.3	0.2
4.56E+02	0.4	0.1
5.70E+02	0.5	0

Table 2. Volume of solutions used in constructing elastase activity standard curve.

contains elevated levels of elastase activity, which has been associated with the debasement of important growth factors and fibronectin necessary for wound healing [3,4]. We suspect a direct correlation lies between the inability of a wound to heal naturally and an increase in elastase activity. Elastase activity is the rate at which this enzyme catalyzes the digestion of elastin, a connective tissue protein, whose role is critical in the healing process.

Serum albumin is expected to be far more concentrated (by at least one order of magnitude) than any of the proteases in wound fluids. Insufficient data have been collected from patients suffering from chronic non-healing wounds to determine actual concentrations of the various proteases. We are interested in the interactions between elastase and wound dressings in the wound site so we quantified the binding interaction between serum albumin and cotton fiber. Serum albumin is the most prominent globular protein found in blood. Even though elastase is present in healing and non-healing wounds, its over-expression in chronic non-healing wounds has been associated with the degradation of important growth factors necessary for wound healing.

It can be said that the local chemistry of the wound fluid is responsible for governing elastase activity. Chemically modifying cotton cellulose while retaining its basic properties, may help to accelerate the healing process of chronic wounds by sequestering over-expressed elastase in wound fluid through the arbitration of elastase activity. This may assist in avoiding the degradation of extracellular elastin and growth factors, thus hastening the healing of chronic wounds.

Our focus is on the design of a synthesized cotton dressing that will enisle, or aid in the reduction of elastase activity as it is expressed in wound fluid to facilitate the body's natural healing ability. The formulation of a wound dressing that potentially has the ability to lower the amount of over-expressed elastase activity merits additional research. Before formulating better dressings, however, a better understanding of the mechanisms involved in cotton-wound dressing and wound-site interactions with key wound-fluid biomolecules merits further exploration.

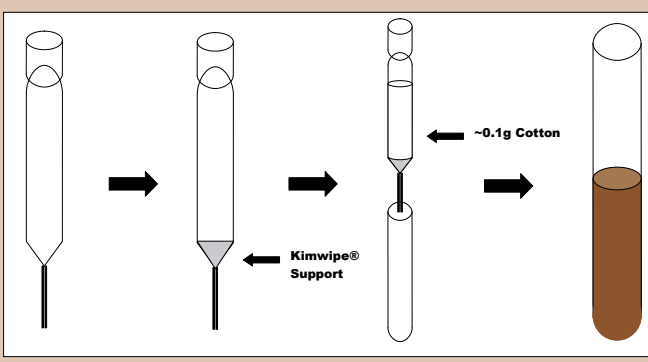

Figure 1. Open-column used in Protein and Enzyme Assay.

MATERIALS AND METHODS

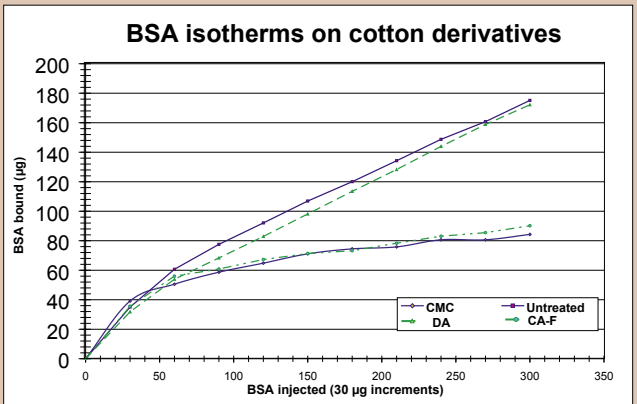

Figure 2. Results of protein assay involving BSA absorption in treated and untreated cotton samples per 0.1g sample. Each data point represents the average of four samples measured.

Protein Assay

Bovine serum albumin (BSA) obtained from Sigma Chemical Co. (St. Louis, MO) was used throughout this study because of its similarity to human serum albumin in structure, molecular weight, and other properties. We believe both forms of albumin have analogous influences on elastase activity. Through the modification of a Bio-Rad protein assay procedure, the following solution was primed: 4:1 dye reagent concentrate (from Bio-Rad Laboratories, Hercules, CA) consisting of 20 mL dye reagent and 80 mL MilliQ water that was processed through a Millipore MilliQ Plus system (from Millipore, Billerica, MA) such that the resistance was greater than 18.2 ohms. A 5 mM buffer solution was made by measuring 0.6055 g of Trizma® (Tris[hydroxymethyl]aminomethane-HCl) salt (Tris) obtained from Sigma Chemical Co. (St. Louis, MO) and placing it in a one-liter volumetric flask brought to volume with MilliQ water. The solution was brought to a pH of 7.4 by adding 1.0 M HCl and using a magnetic stir rod and pH meter. A 0.2 M salt solution was created by adding 2.9220 g of NaCl to a 250-mL volumetric flask and brought to volume with MilliQ water. A 10-mL

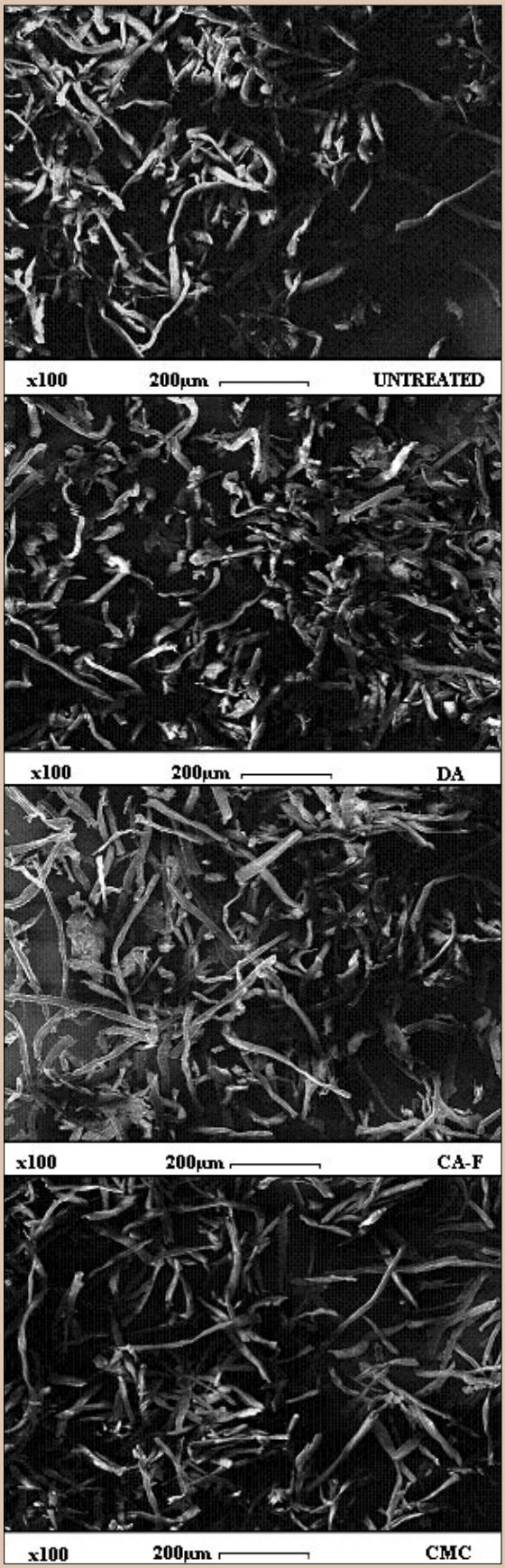

Figure 3. Scanning electron microscope photos of untreated and treated cotton samples.

sample of a 300 µg/mL solution of BSA in 5 mM Tris solution was then prepared by adding 0.003 g of BSA to a 10-mL volumetric flask brought to volume with 5 mM Tris.

The Bradford protein assay [5] was used to measure protein concentrations. Cotton derivatives consisted of carboxymethyl cellulose (CMC), dialdehyde (DAQ), citrate-fructose cellulose (CA-F), and an untreated control sample. All treated and untreated cotton samples were ground and sifted to pass through a –80 mesh screen. All cotton samples were supplied by J. Vincent Edwards at the U.S. Department of Agriculture, ARS Laboratory (New Orleans, LA). Before conducting the open-column assay, a standard curve was created using the volumes of our buffer and BSA solutions found in Table 1.

All experiments were done independently as follows. The masses of all Pasteur pipets used were taken and recorded. A Kimwipe® plug was inserted into each pipet and the mass was recorded. Approximately 0.1 g of cotton sample was placed in each pipet. Actual masses varied and were recorded. Figure 1 depicts the configuration of these open columns.

A collection of 12 test tubes was used per experiment. A blank sample was first made with 0.5 mL salt solution; next a 0.5 mL injection of salt solution was eluted through the open column, followed by the first of ten 100 µL injections of 300 µg/mL BSA in 5mM Tris buffer solution. Elutions were collected in numbered test tubes. Preceding the collection of all eluted samples, 5 mL of the 4:1 dye reagent in MilliQ water was added to each sample and spectrophotometric measurements were taken seven minutes after the dye reagent was added. Spectrophotometric measurements were taken from a Cole Parmer 1200 spectrophotometer. The wavelength was kept constant at 595 nm.

Enzyme Assay

The procedure used to determine the activity of elastase in the presence of treated and untreated cotton samples was similar. The enzyme assay experiment was conducted on only CMC and untreated cotton samples. The 570 µg/mL elastase in 0.2 M NaCl solution was primed by measuring 0.0057 g of porcine pancreatic elastase, which was placed in a 10-mL volumetric flask followed by the addition of 0.0584 g NaCl brought to volume with MilliQ water. A saline solution of 0.15 M Tris, 0.2 M NaCl was made by measuring 0.6055 g Tris and 11.7 g NaCl, which were set in a one-liter volumetric flask brought to volume with MilliQ water and to a pH of 7.4 1.0 M HCl and a magnetic stir rod and pH meter. A 0.6 mM substrate solution was prepared by placing 0.0035 g N-(methoxysuccinyl)-ala-ala-pro-val 4-nitroanalide in a 10-mL volumetric flask and bringing to volume with 100% dimethylsulfoxide (DMSO).

Following a procedure similar to the Bradford protein assay, a standard curve was created using the volumes found in Table 2 of our elastase and saline solutions. Open columns were prepared in the same manner as above. A collection of eight test tubes was used per experiment. A blank sample was first created by pipeting 1.0 mL saline solution in the first of our test tubes. Then, 0.5 mL of the 570 µg/mL elastase solution was injected in each column and subsequent 1.0-mL injections of saline solution were administered. Upon completion of the final elution, a 0.5-mL aliquot of each elution was pipeted into a cuvette. A 100-µL injection of substrate was administered to each cuvette and 20 seconds elapsed between initial exposure and actual reading measurement for each sample in order to incite the release of p-nitroanaline from the enzymatic hydrolysis of MeOSuc-Ala-Ala-Pro-Val-pNA measured at a constant wavelength of 410 nm.

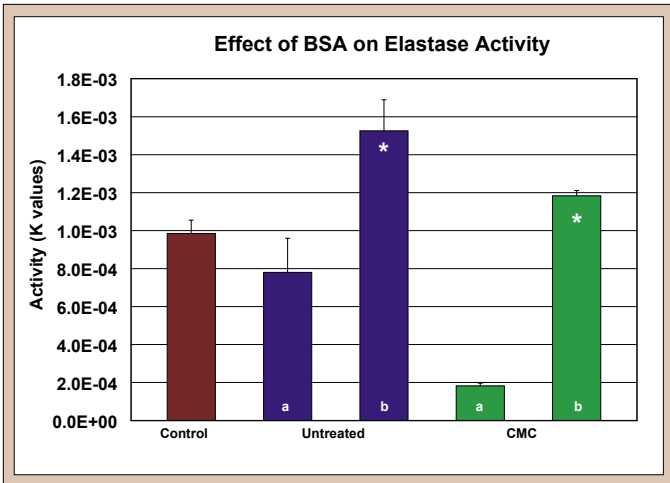

Figure 4. Overall elastase activity of CMC and untreated cotton samples in comparison to initial elastase activity with respect to the presence or absence of BSA. Elastase activity as measured without the presence of BSA (a), elastase activity as measured in the presence of BSA (b). A minimum of two samples per condition were measured.

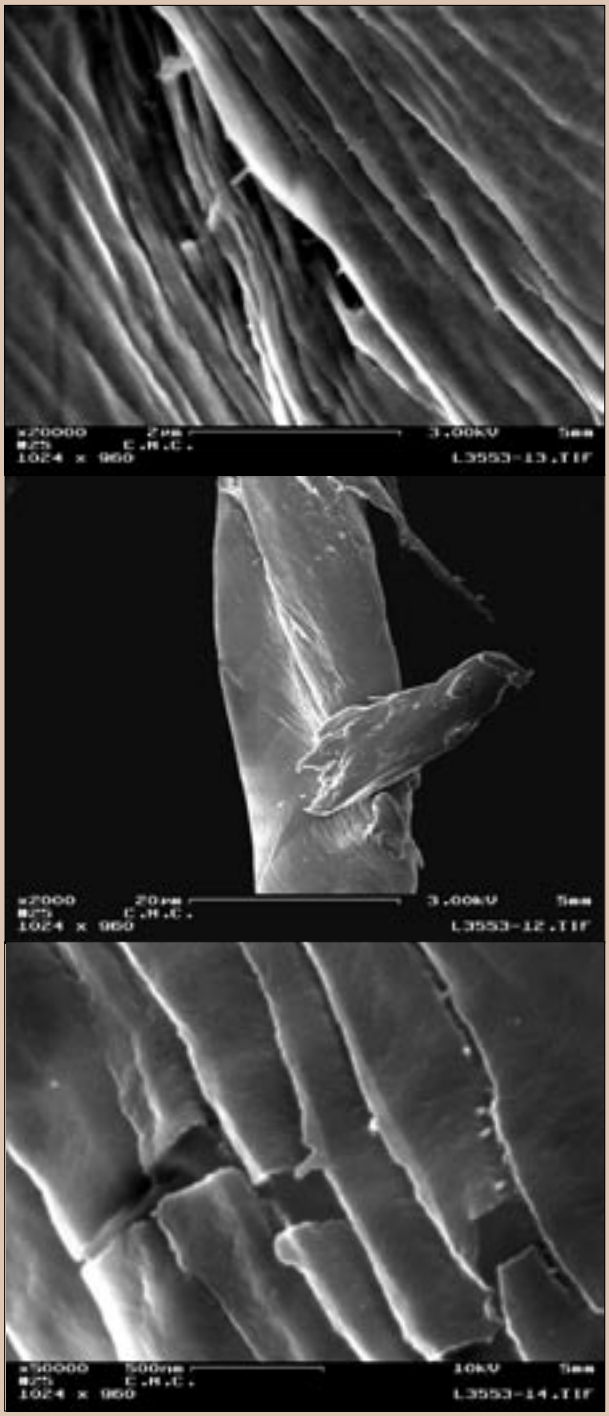

Figure 5. Scanning electron microscope photos of cotton surface depicting porosity of cotton fibers.

Scanning Electron Microscopy Sample Preparation

All four cotton fiber samples were prepared for viewing in a field emission scanning electron microscope in the same manner. A 13-mm scanning electron microscopy (SEM) stub was cleaned and a small piece of graphite tape was placed on the top of the stub. A small amount of the cotton fiber sample was placed on top of the graphite tape. The material that had not adhered to the graphite tape was removed with a small discharge of air from a filtered air source. The sample was placed in an Edwards S150B Sputter Coater under partial vacuum for one hour to allow the small particles to settle on the graphite tape. After the particles had settled, the samples were sputter-coated with carbon for 10 seconds. The samples were removed from the sputter coater and placed in a nitrogen-gas-fed dry box for storage.

The samples were viewed on the LEO 982 FESEM with varying magnifications. The LEO 982 FESEM has resolution capabilities of 1.0 nm at 30 kv. High- and low-magnification images were taken of the particles with the majority of the images being taken of abnormal structures or contamination. The samples were then removed from the FESEM and placed back in the dry box for storage and further examination.

RESULTS AND DISCUSSION

Protein assay results show that all cotton forms (untreated, dialdehyde, CMC, and CA-F) bound about 70 µg BSA per 0.1 g cotton in the first few elutions. The results are depicted in Figure 2. The untreated and dialdehyde samples continued to bind approximately 14 µg BSA per 0.1 g cotton with each subsequent elution. CMC and CA-F bound approximately 4 µg BSA with subsequent elutions. The treated and untreated cotton samples are shown in Figure 3.

One explanation of the results shown in Figure 2 is that the 70 µg of BSA filled pores in the cotton particles and subsequent adsorption was from the accumulation of monolayers (CMC/CA-F) or multilayers (dialdehyde/untreated) of BSA over the exterior of the cotton particles. The inability of the CMC and CA-F to bind additional BSA is likely related to the structure or ionic character of the derivatives. Overall elastase activity increased with elution through both CMC and untreated cotton (Figure 4). Several fractions were collected after injecting elastase and eluting with mild buffer. The earliest eluting elastase fraction was the most active. Typically, no activity was exhibited in the next few fractions. In the seventh fraction, we typically saw additional activity. This confirmed

no activity was present in any of these fractions when elastase was absent. Reasons for the increased activity remain unknown.

CONCLUSIONS

Both CMC and CA-F are negatively charged whereas dialdehyde and untreated cotton samples are neutral. Particles that pass through a –80 mesh sieve are approximately 100 μ in diameter. If we assume all particles were 100 μ in diameter and were perfect cubes, they would have a surface area of approximately 11.1 nm^2/molecule (the density of cotton is 0.3849 g/cm^3). Therefore, it is unlikely the other components of cotton (lignin, protein, etc.) are responsible for this adsorption. The differences in continual binding of BSA to cotton may be related to the orientation of BSA bound to the various derivatives. We suggest the possibility that BSA has a cationic binding site directly opposite a region that resists subsequent binding. If BSA has a region of positively charged amino acids, those are more likely to bind to the anionic cellulose. Then, the remaining exposed surface of BSA would oppose additional binding, by some unknown mechanism. Therefore, by controlling the chemical structure of cotton, bandages can be designed to maximize or minimize the absorption of albumin. All the forms of cotton studied activated elastase. The mechanism by which the activity increased has not been identified. However, future work should be carried out to determine with greater certainty, how the use of cotton can inhibit healing in chronic non-healing wounds by stimulating elastase activity. It may be, for example that albumin will mask this activation effect, or other materials may activate elastase more extensively. Regardless of the answers to these important questions, the results of this study clearly show that bandage materials can play an important role in the biochemistry of wound healing. More research is needed to find the ideal bandage material for chronic non-healing wounds.

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