Enzyme-mediated Crosslinking of Wool. Part I: Transglutaminase

Abstract  Felting shrinkage of wool fabric can be controlled by oxidation and protease treatment, however, strength loss usually results. The Agricultural Research Service (ARS) process, providing bleaching, biopolishing, and shrinkage control by peroxycarboximidic acid oxidation and selective enzyme digestion of wool’s scales can cause 10 to 18% strength loss. After ARS processing 3 to 5% fabric strength was regained with application of transglutaminase (TG). The TG reactivity involves the transferase-mediated, acyltransfer reaction between glutamine and lysine with the formation of carboxylamide groups of peptide-bound glutamine in wool keratin. Changes in the specular reflectance Fourier transform infrared spectra of the amide I and II regions attributed to the functional groups involved in the transamidation reaction provided evidence that cross-linking through TG had occurred in the fabrics. Optical and fluorescence microscopy showed no influence of TG on the anionic charge imparted by peroxycarboximidic acid oxidation and no influence of TG on the scale smoothing or removal of the enzymatic digestion involved in the ARS process. Confocal microscopy revealed the abundant presence of amine groups in the TO-treated fibers. Thus there is evidence that ARS-processed keratin substrates in the solid state can be self-cross-linked and that they have potential for further reactivity.

Key words  ARS process, transglutaminase, specular reflectance FIER, scanning electron microscopy, enzyme-mediated crosslinking of wool

Jeanette M. Cardamone
U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, 600 East Mermaid Lane, Wyndmoor, PA 19038, USA

The ARS process, developed by the United States Department of Agriculture’s Agricultural Research Service (ARS) for whitening, biopolishing, and shrinkproofing wool, is an effective and efficient two-step, chemoenzymatic process involving bleaching, biopolishing, and shrinkproofing. The pretreatment step of this process utilizes stabilized peroxycarboximidic acid, a powerful bleach when applied at pH 11.5 for 30 minutes at 30°C. A subse-

1 The mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture above others of a similar nature not mentioned.


3 Corresponding author: tel: +1 (215) 233 6680; e-mail: jan-cardamone@ars.usda.gov
Enzyme-mediated Crosslinking of Wool. Part I: Transglutaminase

J. M. Cardamone

215

Materials and Methods

Scoured and carbonized wool fibers, 21 micron, 1½″ domestic fleece was supplied by Bollman Hat Company, Adamstown, PA. Worst wool woven fabric (Style #523,
twill weave), and wool jersey knit fabric (Style #532, jersey) were obtained from Testfabrics, Inc., West Pittston, PA. Four fabrics, each 10 g were cut to represent four replicates for each sample condition. All fabric samples were pretreated and treated in an Atlas LP2 Launder-Ometer and Lab Dyeing System in individual canisters containing bath volumes of 400 mL (liquor ratio, 10:1). The pretreatment step of the ARS process bleaches to a high level of whiteness and confers anionic charge to the wool after the application of 3 g/L NaOH, 1 g/L potassium glutonate (Sigma), 3 g/L dicyandiamide (Aldrich), 1 g/L Triton X-114 (7 to 8 ethylene oxide units, 1% solution cloud point, 22°C, Sigma), and 12 mL/L 50% hydrogen peroxide, at pH 11.5, for 30 minutes at 30°C. The subsequent treatment step involves selective enzymatic digestion of the scales of wool. The enzyme bath is constituted as 1.5% on weight of fiber (owf) Esperase 8.0 LTM, a bacterial subtilisin serine protease, 548 TU/mg activity, (Novozymes North America, Inc. Franklinton, NC), Na2SO3 and triethanolamine buffer, pH 8–9, were applied at 45°C for 40 minutes. Alternatively, cysteine (thiol) protease, crude papain from Papaya Latex (Sigma) and/or commercial papain, 200 TU/mg activity Liquipanol T-200™ Enzyme Development Corporation, NY were applied at 2% owf in 20 mmol/L phosphate buffer, pH 7, with 1% owf Triton X-114 at 50°C for 60 minutes after the pretreatment step. After this process we applied Activata™ TG-TI, 38kD, microbial Streptovercillum mobaraense isolate, (Ajinomoto Food Ingredients LLC, Ames, IA) from a fresh bath as 1.5% owf TG from a 1% in 50 mmol/L Tris(hydroxymethyl)aminomethane–HCl at pH 7 for 2 hours at 50°C with liquor ratio 10:1. TG is composed of 1% (w/w) enzyme containing 99% (w/w) maltodextrin with reported activity, 86–135 units/g, where one enzyme unit is defined as the amount causing the formation of 1 μmole of hydroxamic acid in 1 minute at 37°C. The optimal temperature for TG activity is 50–55°C and optimum pH stability is from pH 5 to pH 7.5 [16].

Property Measurements

All fabric samples were conditioned to constant relative humidity and temperature overnight before measurements were taken. Relaxation Shrinkage was measured for dimensional change after pretreatment/treatment according to ASTM D1284, “Relaxation and Consolidation Dimensional Changes of Stabilized Knit Wool Fabrics”. The samples were soaked at room temperature in 0.1% Triton X-114 solution for 4 hours to relax them. They were dried and conditioned before measuring. Felting shrinkage was measured after five machine wash/five tumble-dry cycles using the AATCC Test Method 135-1992, Dimensional Changes in Automatic Home Laundering of Woven and Knit Fabrics. A Kenmore™ washing machine (Sears Company) with 1 cup of Woolite® fabric wash was operated in the delicate wash (permanent press setting) at 35°C for a 30-minute wash/rinse cycle, followed by machine dry (permanent press setting).

A bursting strength tester (SDL International P1000 M229B) was utilized for measuring dry and wet bursting strength, and four measurements were taken from each specimen, two samples were tested and a total of eight measurements were averaged. Whiteness index before and after treatment was measured using the color-insight QC Manager system (BYK-Gardner, Inc.) and ASTM E313 test method. For woven fabrics, tensile strength was tested on Instron Model 1122 Analyzer using 1” × 3” raveled strip with 1000 N load cell, five strips of woven fabric were taken from each specimen. A bursting strength tester (SDL International P1000 M229B) was utilized for measuring the bursting strength of the knitted fabric samples, four measurements were taken from each specimen.

Confocal Microscopy

Confocal optical microscopy was used to determine the anionic charge on the ARS-processed wool yarn cross-sections stained with 0.04 g Rhodamine B (Basic Violet 10, cationic dye), rinsed in tetrachloroethylene, and dried. The cross-sections were prepared on the Micro No. 200-A Microtome (Micro Instrument, Marshfield Hills, MA) using collodion embedding medium (Mallinckrodt, Paris, KY). Cross-sections of 5 μm thickness were visualized as confocal fluorescent images by a Leica TCS Confocal System, equipped with an HCX PL40X 1.25 NA lens using an excitation wavelength of 488 nm and an emission wavelength of 540–580 nm.

Confocal fluorescence microscopy was used to determine the effect of TG on wool. The fluorochromes, fluorescein, cadaverine and fluorescein-5-EX, succinimidyl ester (Invitrogen, Molecular Probes, Inc., Eugene, OR), excitation 470–500 nm and emission 510–535 nm, were used to treat wool fibers for the detection of available amine groups needed for TG transcatalysis crosslinking of wool keratin. Stock solutions of each fluorochrome were prepared in sodium bicarbonate buffer (10 mg/mL) at pH 8.3. Each 2-mg wool sample was treated at room temperature with a 250 μL aliquot sample of fluorochrome for 5 hours. After rinsing individually in the buffer and rinsing several times with vortexing, the samples were extracted from clear solutions. After air drying they were mounted in Collodion™ (J.T. Baker, Phillipsburg, NJ) and cross-sectioned to 5 μm thickness on a Micro No. 200-A Microtome (Micro Instrument Division of KFK Machine, Marshfield, MA). Confocal images were collected from a Leica Microsystems Inc. microscope equipped with fluorescence capability, Model: TCS-SP Confocal Laser Scanning Microscope, manufactured by Leica Microsystems Inc. (Exton, PA) with excitation at 488 nm at 15% intensity and emission at 525 to 625 nm at 63× magnification in water mount using a 63x magnification water mount.
Results and Discussion

Physical Properties

Blank samples (fabric in water medium without chemicals under reaction conditions) and control samples (as received) were included in the study. Relaxation shrinkage results from induced stress and strain imparted in yarn and/or fabric manufacture and in processing, especially finishing. Figure 3a and b shows the contribution relaxation shrinkage can make to overall shrinkage that includes felting shrinkage. It must be mentioned that the ARS process specifically controls felting shrinkage; that is, the dimensional stability of relaxed fabrics when they are subjected to laun-

Figure 3 (a–c) Effects of TG on shrinkage and strength of wool fabrics with Esperase™ used in ARS-processing. (a) shrinkage of twill woven fabric. (b) shrinkage of jersey knit fabric. (c) strengths of woven and knit wool fabrics.
dering. TG applications to ARS wool processed wool fabrics showed improvement in fabric strength in Figure 3c.

Note the high contribution that relaxation shrinkage makes to the overall shrinkage, the higher relaxation shrinkage in the knit fabric relative to the woven, and the effectiveness of the ARS process to control shrinkage to 3% without the influence of TG. After applying TG to ARS processed knit and woven fabrics, approximately 4 to 5% strength was regained relative to the value of either blank or control fabrics.

Similarly, Liquipanol T-200™ papain was applied in the ARS process in place of Esperase™ enzyme in Figure 4a and b.

After applying TG to ARS processed knit fabrics treated with Liquipanol T200™, no effect on shrinkage control was observed; however, approximately 3 to 4% strength was regained relative to the value of either blank or control fabrics.

**Confocal Microscopy**

The advantage of using confocal microscopy is that wool in its natural state can be observed as in-depth optical sections by longitudinal and transverse optical sectioning without the dehydration and coating required in scanning electron microscopy. Two channels, optical and fluorescence can be utilized. When fluorochromes are used to stain the specimen, the internal; structure can be revealed to indicate routes of penetration into and selective changes of fiber structure. Fluorescent Rhodamine B, cationic dye, was used to show diffuse anionic charge distribution throughout the untreated wool fiber due to sulfur's electron-pair distribution in Figure 5a. By contrast, after ARS oxidative bleaching, in Figure 5b, Rhodamine B was strongly and selectively absorbed by sulfoxylate groups of the cuticle to indicate localization of anionic charge on the fiber surface. Apparently the dye did not penetrate into the cortex.

We compared fibers extracted from the yarns of untreated and ARS-treated fabric that had the subsequent TG treatment described above using fluorescein cadaverine and fluorescein-5-EX, succinimidyl ester fluorochromes. These fluorochromes, as amine-reactive reagents, can label non-protonated aliphatic amine groups, including terminal amines and the ε-amino group of lysines.

We used Fluorescein as an amine-reactive probe to react with lysine’s ε-amino group. After exposure to TG we applied the fluorochrome to detect whether these amino groups were affected by TG-assisted inter- and/or intramolecular crosslinking. In Figure 6a there is bright, uniform fluorescence in untreated wool and in Figure 6b there is dull, nonuniform fluorescence to indicate that TG was effective in mediating transamidation through amine functionality.
FTIR Spectroscopy

In Figure 7a–d infrared spectroscopy was used to probe protein molecular structure at the secondary level (the underlying structure of alpha-helical keratin consisting of a complex pattern of covalent bonds). Amide I (1700–1600 cm\(^{-1}\)), II (1500–1600 cm\(^{-1}\)), and III (1200–1320 cm\(^{-1}\)) absorbances were examined within the peptide CONH group. Amide III absorbances are relatively the strongest and they correspond to the C=O stretch weakly coupled with the C—N stretch and the N—H bending, whereas the amide II region represents C—N stretch strongly coupled with N—H bending, and the amide III region is N—H in-plane bending coupled with C—N stretching and also includes C—H and N—H deformation vibrations \[18, 19\]. Based on the pathway for TG transamidation shown in Figure 1 this approach is particularly applicable. Derivative spectroscopy provided qualitative information on discrete peak absorptions and curve-fitting provided quantitative analysis to make relative comparisons among wool knit fabrics: untreated (CK2), ARS-treated with Esperase 8.0 L\(^{TM}\) (K2), and K2 followed by treatment with TO (K6). The aliphatic amide in glutamine should show the “amide I” strong C=O stretching at 1650–1690 cm\(^{-1}\), amide II band, NH\(_2\) bending at 1640–1600 cm\(^{-1}\) and the amide III band, C—N stretching at 1420–1405 cm\(^{-1}\). The IR contributions for —NH\(_2\) are weak absorbances within the range of 3550–3000 cm\(^{-1}\). Other spectral regions of interest, 1120–1020 cm\(^{-1}\) represents sulfur-oxygen vibrations attributed to cystine oxidation in the bleaching step of the ARS process.

The second-derivative spectra were used to determine the number of bands and their positions in order to apply curve-fitting to resolve the individual bands that fit the spectral area of interest. This process involved iterating band height, position, and bandwidth at half-height to determine individual parameters in order to achieve the best Gaussian-shapes curve that fit the original spectra. The display of the family of bands comprising the overall band allows for analysis of the individual component bands.

In Figure 7e the resolved IR bands #1 to #12 were normalized to band #9 that showed no absorption change among the samples, CK2, K2, and K6. Note that in Figure 7e the absorbances of CK2 and K2 showed no change in the amide region after the application of the ARS process. However, by following the ARS process with TG treatment (K6 fabrics) there were increases in the amide bands #2, #3 and #4 (amide I and II).

In Figure 7f, the ARS treated fabric, K2, showed the development of sulfoxide bands, as expected from the oxidation of wool, that is, formation of —SO\(_2\)-S— (1118 cm\(^{-1}\)), —SO\(_3\)- from cysteic acid (1040 cm\(^{-1}\)), and —S—SO\(_3\)- (1024 cm\(^{-1}\)) \[20, 21\].

Conclusions

The transamidating activity of TG was observed in the self-crosslinking of wool. TG was effective for mediating in-situ crosslinking of ARS-processed wool fabric when applied from fresh baths at 50°C for 2 hours at pH 7. Evidence of the reaction of transamidation, the crosslinking wool’s amino acids, glutamine and lysine, to form ε-(glutamyl)lysine bonds, was apparent from images from fluorescence confocal microscopy. Yet in terms of potential reactivity, after TG-mediated crosslinking, the persistence of fluorescence indicated the presence of available amine groups. Confocal microscopy utilizing cationic fluorescent dye, Rhodamine B, showed that the anionic surface charge of ARS-processed fibers had been preserved after treatment with TG with no impediment to further reactivity. Scanning electron microscopy images (not shown) indicated no apparent structural changes. The TG-treated fabrics remained biopolished with no alteration in scale smoothing or removal and the structural integrity of the fibers remained intact. A moderate increase of 3 to 5% in fabric strength was consistent with TG-mediated crosslinking of wool.

Examination of the amide I and II regions of the infrared spectra provided substantive evidence that cross-linking through TG had occurred in the fabrics. In the expanded IR regions of the spectra (1750–1350 cm\(^{-1}\)) there were discrete changes in the amide I and II bands when control (CK2), ARS-processed (K2), and ARS-processed and TG-treated (K6) samples were compared. Specifically, the band differences in the amide I region, 1750–1650 cm\(^{-1}\),
in Figure 7b pointed the way to further examination by second-derivative spectral analysis for isolating the distinctive bands of interest in Figure 7c. The amide I band signaled changes within the CONH group of glutamine, the site of proposed TG cross-linking mediation. After peak resolution through curve-fitting in Figure 7d of the K6 spectrum and normalization with the 1250 cm\(^{-1}\) band as reference, it was apparent that significant changes had occurred in the...
Enzyme-mediated Crosslinking of Wool. Part I: Transglutaminase J. M. Cardamone 221

salient amide I and amide II absorbances. These changes were then attributed to the functional groups involved in the proposed transamidation reaction of enzyme-mediated TG crosslinking of wool.

Evidence is therefore provided that keratin substrates in the solid state can be self-cross-linked and that there is the potential for the self-cross-linking of other keratinaceous materials.

Acknowledgements
The author acknowledges the technical support of Guoping Bao for experimentation and William A. Stanert for collecting confocal images and Fourier transform infrared spectra.

Literature Cited