Viscoelastic Behavior of Formaldehyde and Basic Chromium Sulfate-Crosslinked Collagen

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ABSTRACT: Rat tail tendon (RTT) collagen has been crosslinked with 1% basic chromium sulfate (BCS) at pH 3.2, and 2, 4, 8, and 10% formaldehyde (HCHO) at pH 5 and pH 8. The viscoelastic behavior (such as stress relaxation behavior for BCS and HCHO-tanned RTT) has been studied in water and 6*M* urea at different temperatures. The total rate of relaxation has been divided into fast and slow components, and computed using the two-term model and nonlinear least-squares fit. The rate of relaxation for crosslinked RTT is less than the native one. Activation energy at absolute zero has been computed using $k = AT^m e^{-E_0/RT}$ and nonlinear least-squares fit. The activation energy increases for crosslinked RTT than the native one. This is consistent with the observed rate constant values. This may be due to the additional stability imparted to RTT collagen by coordinate covalent and covalent crosslinks through BCS and HCHO, respectively. © 1999 John Wiley & Sons, Inc. J Appl Polym Sci 71: 2245–2251, 1999

Key words: basic chromium sulfate; formaldehyde; rate of relaxation; activation energy

INTRODUCTION

Crosslinks in collagen can be either intramolecular or intermolecular. In either case, a principal function of crosslinks is the stabilization of an organized structure. Chemical processing of skin is known to influence and alter the matrix stability. One of the commonly adopted strategies to the stabilization of collagenous matrices for industrial application is to introduce additional (artificial) crosslinks. Chemical reagents, in particular, bifunctional aldehydes like glutaraldehyde, have been found to participate in the formation of new crosslinks.¹ The use of formaldehyde to introduce new crosslinks in collagen has long been known. Formaldehyde has been used as a tanning agent in the leather and wool industries. In an industrial application like collagen in the form of leather, chromium salts are extensively used. It is believed that the treatment of skin with salts of chromium introduces coordinate covalent crosslinks involving a -COO-Cr-OOC- (Scheme 1, ref. 2) type bridge, and formaldehyde introduces a -C-N-C-N-C- or -C-N-C-O-C-N-C- (Scheme 2, ref. 7 and Scheme 3) type of covalent crosslink.²⁻¹²



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The interaction of formaldehyde with side chain amino groups of collagen is followed by further

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condensation with amide and guanidyl group to form methylene bridges, and is given in Scheme 2. The reaction of formaldehyde with the amino group to give the hydroxymethyl group is highly reactive, and crosslinking can occur with a second hydroxymethyl group to give an oxomethylene bridge and is given in Scheme 3.

$$\operatorname{R--\!NH}_2 + \operatorname{CH}_2\operatorname{O} \rightarrow \operatorname{R-\!-\!NH}-\!\operatorname{CH}_2\operatorname{OH}$$

$$\begin{array}{c} \text{R---NH---CH}_{2}\text{OH} + \text{H}_{2}\text{N----}\overset{|}{\text{C}}\text{---R'} \rightarrow \\ & \text{R----NH---CH}_{2}\text{-----------------------R'} \\ & \text{H} \end{array}$$

$$\begin{array}{cccc} \mathrm{R-\!\!-\!NH-\!\!-\!CH_2OH} + \mathrm{H_2N-\!\!-\!C-\!\!-\!R''} \rightarrow \\ & \parallel \\ \mathrm{NH} \\ \mathrm{R-\!\!-\!N-\!\!CH_2\!\!-\!N-\!\!C-\!\!-\!R''} \\ & \parallel \\ \mathrm{H} & \mathrm{H} & \mathrm{NH} \end{array}$$

Scheme 2

$$\label{eq:R_NH_2} \begin{array}{l} \text{R}_\text{NH}_2 + \text{CH}_2\text{O} \rightarrow \text{R}_\text{NH}_\text{CH}_2\text{OH} \\ \\ \text{R}_\text{NH}_\text{CH}_2\text{OH} + \text{OHCH}_2_\text{NH}_\text{R}_1 \rightarrow \\ \\ \\ \text{R}_\text{NH}_\text{CH}_2_\text{O}_\text{CH}_2_\text{NH}_\text{R}_1 \end{array}$$

Scheme 3

Whereas chrome-tanned collagen fiber is known to resist thermal shrinkage to relatively higher temperature, formaldehyde is believed to impart crosslinks of varying thermal stability. Although shrinkage temperature provides the measure of thermodynamic property, it is very difficult to design the experiments that measure the thermodynamics of crosslink formation. One of the unique physical properties of collagen is the viscoelasticity.¹³ Viscoelastic models have been applied to predict the behavior of collagen. The dimensional and matrix stability of collagen have been studied using the viscoelastic properties like stress-strain, creep, and stress relaxation behavior. Many viscoelastic models need to envisage a spectrum of relaxation times, depending on the molecular constituents and heterogeneity of material.^{14–17} It has been reported that crosslinking, hydrogen bonding, temperature, and solvents influence the viscoelastic behavior of collagen.^{18–20} Stress relaxation behavior of collagenous tissues and rat tail tendon (RTT) has already been investigated in detail.^{21–23}

MATERIALS AND METHODS

Sample Preparation

Collagen fibers were teased out from tails of 6-month-old male albino rats (Wistar strain). The area of cross-section of the fibers was determined by taking the diameter of the fiber using a filer micrometer attached to the optical microscope in the wet condition. After cleaning, the RTTs were tanned with 1% basic chromium sulfate (BCS) at pH 3.2, and 2, 4, 8, and 10% formaldehyde (HCHO) at pH 5 and pH 8, and kept overnight. It is used to crosslink RTT fibers with 1% formaldehyde solution at pH 8.24 The isoelectric point of collagen is \sim pH 7. While liming the hide, deamidation takes place; there is a decrease in amide nitrogen and an increase in the number of free carboxylic groups that, in turn, cause an increase in the negative charge. Thus, the isoelectric point of collagen is shifted toward pH 5.3. The hydrothermal shrinkage temperature of HCHO and BCS tanned RTT fibers were studied using the Instron testing machine.

Experimental Methods

Stress relaxation experiments have been chosen to calculate the activation energy values. It is possible to calculate the activation energy values from the relaxation times for crosslinked collagen fibers at different environmental conditions as a function of temperature. Stress relaxation experiments were conducted in an Instron testing machine model 1112 with a liquid cell container for HCHO and BCS-tanned RTT fibers in water medium at 298, 313, 323, 333, and 353 K. The fiber is strained up to a 20% strain level; thereafter, the strain is maintained constant and stress decay was monitored for 3 h. From the load-time graph, the values of σ/σ_0 (where σ is the stress at time t and σ_0 is the initial stress) as a function of time



Figure 1 Stress-relaxation behavior of native (n) and formaldehyde-crosslinked (F) RTT in water at 298 K and 323 K.



Figure 2 Experimental and calculated values of σ/σ_0 against time for formal dehydetanned RTT in water at 298 K.

	Formaldehyde-Tanned RTT		Native RTT	
Temperature (K)	$k_1 \times 10^{+3} \ ({ m s}^{-1})$	$k_2 \stackrel{\times}{\times} \stackrel{10^{+5}}{}_{({ m s}^{-1})}$	$\overline{k_1 \times 10^{+3}}_{({ m s}^{-1})}$	$k_2 \stackrel{ imes 10^{+5}}{ ext{(s}^{-1})}$
298	3.6 ± 1.0	1.5 ± 0.5	$4.5 \pm 0.8 \\ 18.5 \pm$	4.0 ± 1.0
323	4.0 ± 2.0	3.6 ± 0.5	0.4	54 ± 3.0
343	5.0 ± 0.4	6.0 ± 0.1		_
353	15.4 ± 0.4	6.0 ± 0.4	—	—

 Table I
 Comparison of the Rate of Relaxation (Both Fast and Slow) for Native and

 Formaldehyde-Tanned RTT in Water Medium at Different Temperatures

Values are means \pm SD of six determinations.

have been plotted. Values of σ/σ_0 have been better treated using the equation

$$\sigma/\sigma_0 = A_0 e^{-k_1 t} + B_0 e^{-k_2 t} \tag{1}$$

(where A_0 and B_0 are the preexponential factors, and k_1 and k_2 are fast and slow rates of relaxation) rather than a single-term equation

$$\sigma/\sigma_0 = A_0 e^{-k_1 t} \tag{2}$$

Values of the preexponential factors and exponential factors for native HCHO and BCS-tanned fibers at different temperatures were computed using nonlinear least-squares fit using a Hewlett– Packard workstation and standard packages.²⁵

RESULTS AND DISCUSSION

The stress relaxation profile of formaldehyde (with 2% solution at pH 5)-treated RTT fibers has been obtained in water medium at different temperatures. Comparison of native and HCHO-tanned RTT fibers at 298 and 323 K are given in Figure 1. Whereas untanned RTT fibers under-



Figure 3 Stress relaxation behavior of native and BCS-tanned RTT in water at 298 K.



Figure 4 Stress-relaxation behavior of BCS-tanned RTT in water and urea (6M) at 298 K.

went major deformation > 328 K, stress relaxation of HCHO-treated fibers shows an enhanced dimensional stability against thermomechanical stress. Native RTT fibers relax faster than HCHO-tanned fibers. This may be due to the additional crosslinks introduced by HCHO that tend to hold the fiber from relaxing faster. Although the stress relaxation behavior of tanned fibers is slow, the observed experimental data fit the twoterm model more closely with the experimental values, as seen in Figure 2.

Both fast and slow rates of relaxation $(k_1\times10^{+3}\,{\rm and}\,k_2\times10^{+5}\,{\rm s}^{-1})$ for HCHO-tanned and

native RTT fibers at different temperatures are given in Table I. The rate of relaxation decreases in HCHO-tanned RTT fibers than native RTT fibers. This decrease is more pronounced at 323 than at 298 K. Thus, in HCHO treatment, a long-range order seems to increase in RTT, thereby causing enhanced thermomechanical stability in the temperature range of 293 to 348 K.

The comparison of the stress relaxation profile of BCS-tanned and native RTT fibers at 298 K in water medium is given in Figure 3. It is obvious that native RTT fibers relax faster than the BCS

Temperature (K)	BCS-Tanned RTT		Native RTT	
	$k_1 \times \frac{10^{+3}}{(\mathrm{s}^{-1})}$	$k_2 \stackrel{ imes 10^{+5}}{ ext{(s}^{-1})}$	$k_1 \stackrel{\times}{\underset{({f s}^{-1})}{\times}} 10^{+3}$	$k_2 imes 10^{+5} \ { m (s^{-1})}$
298	2.0 ± 0.6	3.0 ± 1.0	$4.5 \pm 0.8 \\ 18.5 \pm$	4.0 ± 1.0
313	4.5 ± 1.0	5.0 ± 0.5	0.4	54 ± 3.0
333	9.5 ± 4.0	6.0 ± 0.1		_
363	9.2 ± 2.0	8.5 ± 1.4	_	_

 Table II
 Comparison of the Rate of Relaxation (Both Fast and Slow) for BCS-Tanned and

 Native RTT in Water Medium at Different Temperatures

Values are means \pm SD of six determinations.

(Both Fast and Slow) for Native and BCS- Tanned RTT in 6 <i>M</i> Urea Solution at Room Temperature		
	Rate of Relaxation	

Table III Comparison of the Rate of Relaxation

	Rate of Relaxation		
Specification	$k_1 \stackrel{ imes 10^{+3}}{ ext{(s}^{-1})}$	$k_2 imes 10^{+5} \ ({ m s}^{-1})$	
BCS-tanned RTT	4.0 ± 0.6	3.0 ± 1.0	
Native RTT	18 ± 8.0	39 ± 5.0	

Values are means \pm SD of six determinations.

tanned ones. The stress relaxation profile of BCStanned RTT fibers in water and 6M urea solution at 298 K is given in Figure 4. BCS-tanned RTT fibers relax faster in urea than in water. This may be due to destabilization introduced in the matrix by breaking the intermolecular hydrogen bonds in the presence of urea solution.^{26,27}

The comparison of the rate of relaxation of both fast and slow $(k_1 \times 10^{+3} \text{ and } k_2 \times 10^{+5} \text{ s}^{-1})$ for native and BCS-tanned RTT fibers at different temperatures in water medium, and the comparison of BCS-tanned and native RTT fibers in 6M urea at 298 K are given in Tables II and III. Chromium-tanned RTT fibers resist dimensional changes considerably, as seen from the decrease in the values of rate constants at 323 K. These observations indicate that chromium may participate in increasing the long-range order through intermolecular crosslinks, thereby increasing thermal stability beyond 323 K. Increased longrange order in chromium-tanned RTT fibers seems to influence such a phase transition. In the case of native RTT fibers, a phase transition seems to occur at 333 K. This could explain, in

part, the lower relaxation rate of chromiumtanned RTT fibers at 323 K in the aqueous medium. Crosslinking induced by chromium in RTT fibers leads to enhanced dimensional stability, with the result that the relaxation rate of chromium-tanned RTT fibers in 6M urea at 298 K is two times faster for the k_1 path and independent of urea concentration for the k_2 path. Although crosslinking processes associated with chromium tanning may involve coordinate covalent bonds, significant stability to the secondary structure of collagen seems to be added by chromium tanning. Because Arrhenius plot is nonlinear, activation energy values at absolute zero were computed using the equation²⁸ with nonlinear least-squares fit and the Hewlett-Packard workstation of standard packages²⁵

$$k = AT^m e^{-E_0/RT} \tag{3}$$

(where A is the preexponential factor and E_0 is the activation energy at absolute zero).

Two relaxation events associated with rate constants k_1 and k_2 have been identified for both HCHO and BCS-tanned RTT fibers. Activation energies $E_{\,0}$ for the $k_{\,1}$ path and $E_{\,0}^{\prime}$ for the $k_{\,2}$ path are given in Table IV. The observed activation energies E_0 and E'_0 for HCHO and BCS-tanned RTT fibers are higher than untanned RTT fibers. This is consistent with rate constant values. Although HCHO and BCS tanning may implicate changes in tertiary and quaternary structures, an indirect influence on the protein-water interaction and, consequently, on the secondary structure of collagen may seem to be influenced. Because stabilization against thermomechanical stress under hydrothermal conditions is being investigated, solvational energies are of impor-

Table IV Activation Energy Values at Absolute Zero for Native, Formaldehyde, and BCS-Tanned RTT Computed Using Both $k_1 \times 10^{-3}$ and $k_2 \times 10^{-5} \text{ s}^{-1}$

	Activation Energy (kcal mol^{-1}) at Absolute Zero		
Specification	Using $k_1 \times 10^{+3}$ (s ⁻¹) E_0	Using $k_2 \times 10^{+5}$ (s ⁻¹) E'_0	
Native RTT Formaldehyde-tanned RTT BCS-tanned RTT	$\begin{array}{r} 28.0 \pm 3.0 \\ 40 \pm \ 7.0 \\ 37 \pm \ 6.0 \end{array}$	$33.6 \pm 4.0 \ 41.5 \pm 4.0 \ 48 \ \pm 6.0$	

Values are means \pm SD of six determinations.

tance. It is known that HCHO-tanned collagen is more extensively desolvated, thus drying and rewetting processes pose more serious problems. After the chromium tanning process, collagen exhibits generally higher hydrothermal stability. It is noteworthy that activation energy for the k_2 path for chromium-tanned RTT collagen is significantly higher than that for native and HCHOtanned RTT fibers.

CONCLUSIONS

- 1. Stress relaxation processes are slower in tanned RTT collagen fibers than the native one. This is due to the additional crosslinks introduced by the tanning agents that tend to hold the fiber from relaxing faster.
- 2. The decrease in the rate of relaxation (both fast and slow) for HCHO and BCS-tanned RTT fibers is significant in the higher temperature range.
- 3. Although stress relaxation processes are slower for tanned fibers, the observed experimental data fit the two-term model.
- 4. The higher activation energy for BCStanned collagen indicates that the longrange order is increased through coordinate covalent crosslinks.

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