Soluble Collagen Approach to a Combination Tannage Mechanism

by

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Abstract

Although complex salts of Cr^{III} sulfate are currently the most effective tanning agents, salts of other metals, including aluminum, have been used either alone or in combination with vegetable tannins or other organic chemicals. In the present study, the interactions of aluminum sulfate, and quebracho or chestnut tannins with collagen were investigated. A model system was devised to use soluble collagen in one compartment of an equilibrium dialysis cell and solutions of mineral or polyphenolic tanning agents in the other compartment. This study, by focusing on the effects of tanning agents on soluble collagen, rather than on intact hide, or powdered hide, gives a somewhat different perspective on the tanning process. The extraction of water from the collagen solution in the formation of aluminum/tannin complexes suggests that the lowering of water activity around the collagen may play a role in stabilizing a collagen/tannin/aluminum tannage. The most interesting finding is that aluminum which has little effect on collagen helical structure and stability does appear to connect collagen molecules in some manner to produce high molecular weight species that do not separate under the conditions of SDS PAGE. Comparison of the interactions of various combinations of minerals and vegetable tannins with collagen is expected to provide insight into a more generalized mechanism for tanning.

Introduction

Tanning is a multistep process whereby a perishable animal hide or skin is converted into leather, which resists microbial attack and may last indefinitely. Biologically, the skin is connective tissue, comprised mainly of the extracellular matrix, a fibrous collagen structure. Tanning stabilizes the collagen matrix, protecting it against heat, water, and microbes. More than a hundred years ago, the salts of chromium (Cr^{III}) emerged as nearly ideal tanning agents for the rapid production of fine leathers. Over the past century, chromium sulfate came to be the most widely used and studied tanning agent. Although it is not always recognized, most chrome-tanned leather is retanned with vegetable or synthetic tannins, thus making it combination tanned.

In the early literature on combination tanning, Fein *et al*.[1] observed that for chromium/glutaraldehyde combinations, whether used sequentially or in a mixture, the two agents appeared to act independently with each imparting specific characteristics to the leather. Combination tannages that employ aluminum rather than chromium have been known for more than a thousand years.[2] The work of Hernandez and Kallenberger[3] noted the effect, of the order in which aluminum and vegetable tanning agents were applied, on the shrinkage temperature of the resulting leather, with the implication that aluminum was reacting with the tannin rather than with collagen. Research on combination tannages, usually vegetable tannins combined with a mineral or an aldehyde, has increased dramatically in the 21st century. For the most part, these studies are performed on sheep or goatskins or bovine hides and always produce leather.

Previously, we used the soluble collagen model to examine the interactions of Cr^{III} with collagen in dilute acetic acid (HAc),[4] and the effects of neutral salts[5, 6] or $Al_2(SO_4)_3$[7] on the stability of the collagen triple helix. In those studies, ^{13}C NMR spectroscopy suggested that Cr^{III} interactions were mainly with the carboxyl groups of aspartic and glutamic acids in collagen[4] and Al^{III} interactions were primarily with glutamic acid.[7] Changes in the temperature coefficient of the helix to coil transition for soluble collagen, estimated from the circular

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dichroism (CD) spectra, showed a destabilizing effect of Al^{III} at 30°C . Li *et al*.[8] reported decreasing viscosity for soluble collagen in the presence of Al^{III}, particularly above 30°C . In this study, a soluble collagen model is used to obtain information at the molecular level on the effects of aluminum sulfate, in combination with condensed and hydrolyzable vegetable tannins, on the individual collagen molecules.

EXPERIMENTAL

Materials

Solubilized adult bovine skin collagen (type I), was purchased from Inamed (Fremont, CA) as PureCol™, a sterile solution in 0.01 N HCl at a concentration of 3 mg/mL. Tannery grade chestnut and quebracho tannins were obtained from Hermann Oak Leather (St. Louis, MO). Other chemicals were reagent grade.

Experimental Design

Collagen samples were dialyzed in 10,000 MW cutoff dialysis cassettes (Pierce, Rockford, IL) against the appropriate solvent, 0.05 M HAc or the experimental buffer, overnight at 4°C, to remove small peptide degradation products and to assure an equilibrium solvent composition. Crude tannins were washed with hexane to remove oily residues, then filtered and dried overnight under vacuum to remove hexane. Hexane washed tannins were extracted with water at 60°C for one hour, then centrifuged for 20 min at 9000 rpm. The supernatant was diluted with buffer and used in experiments.

A 1 mL equilibrium dialysis cell (Bel-Art Products, Wayne, NJ) was used as a reactor for the study of interactions between soluble collagen and tanning agents. The cell is comprised of two acrylic blocks, each with a 1 mL half-cavity in the center. A dialysis membrane is placed across the cavity between the two blocks, and the assembly is held together with screws and wing nuts. Collagen solution is placed in one compartment, through a screw-sealed port for inserting and removing solutions, and a solution of the tanning agent of interest is placed in the other compartment. The assembly was incubated at 20 – 25°C, 100 rpm for 24 hours in a Mini IncuShaker™ model SH1000 (Southwest Science, Robeling, NJ). After 24 h, the tanning reagent was removed. For sequential studies, an aliquot of the treated collagen solution was removed for analysis, and the other side of the cell was filled to the new level of the collagen solution with the second tanning agent.

Spectroscopy and Thermal Stability

Samples, 300 mL in volume, containing 1 - 2 mg/mL collagen were placed in 1 mm pathlength cuvettes. The cuvettes were made of far-ultraviolet transparent quartz, and fitted with Teflon stoppers to prevent evaporation during melting experiments. The ultraviolet (UV) spectra of the samples were scanned (Model 14 Spectrophotometer, AVIV Biomedical, Lakewood, NJ) at ambient temperature from 300 nm to 200 nm against buffer. The concentration of collagen in colorless solutions was estimated from the absorbance at 218 nm using the molar absorptivity (e = 883,129 cm^{-1}-L-$mole^{-1}$) determined by Na.[9] For solutions that were colored by the tanning agent, concentration was estimated by the appropriate dilution factor from the stock solution.

Because the slow unwinding of the triple helix in dilute solutions of collagen begins below 30°C , the stoppered cuvette containing the sample was refrigerated at 4°C for at least 12 h to maximize the stabilization of the helical conformation. The hydrothermal stability of collagen helical structure was then determined by following the change in the CD signal at 223 nm (Model 420 Circular Dichroism Spectrometer, AVIV Biomedical, Lakewood, NJ) with increasing temperature. The instrument was programmed with a scan-melt-scan algorithm so that the sample was scanned at 10°C from 250 nm to 200 nm in 1 nm steps using a 2 sec time constant. Melting curves were obtained by recording the CD signal at 223 nm every 0.5 deg between 10°C and 60°C with a time constant of 10 sec and a heating rate of 7°C per hour. A final scan was made after the sample was cooled to 10°C . The CD signal was read in mdeg and converted to molar ellipticity by equation 1.

$$[\theta]_\lambda = \theta_\lambda/ncd, \text{ deg cm}^2 \text{ dmol}^{-1} \quad (1)$$

Where n is the number of amino acid residues in the protein chain, c is the molar concentration, and d is the pathlength in millimeters. Pretransition and denaturation temperatures (T1 and T2) were obtained from the derivative of the melting curve.

Electrophoresis

Aliquots, 100 μL, were removed from the protein containing side of the cell, lyophilized, and dissolved in electrophoresis buffer (1mM EDTA, 2.5% SDS, 5% β-mercaptoethanol and 0.01% bromophenol blue) for analysis by SDS-PAGE (polyacrylamide gel electrophoresis in sodium dodecyl sulfate). The samples were heated at 40°C for 4 h. Separation was achieved on 4-15% gradient gels using a Phast-gel system (GE Healthcare Life Sciences, Piscataway, NJ). A broad range SDS-Standard (Bio-Rad, Hercules, CA) containing nine proteins ranging in size from 6,500 to 200,000 Daltons was included on each gel. Gels were stained with Coomassie Brilliant Blue R.

Collagenase Resistance

Collagenase resistance was determined by a modification of the Mandl method.[10, 11] Duplicate aliquots (100 μL) of treated collagen were transferred from the protein side of the equilibrium dialysis cell to borosilicate tubes, and lyophilized. The dry samples were suspended in 2.5 mL 0.05 M TES

(tris (hydroxymethyl)-methyl-2-aminoethane sulfonate) buffer, pH 7.5, containing 0.36 mM calcium chloride, the tubes were capped, and incubated in the Mini IncuShaker™ at 37°C , 100 rpm for 30 min. After the initial incubation, 100 µL collagenase, 1 mg/mL in TES, was added to one tube of each pair while the other received 100 µL TES without collagenase. All tubes were then incubated for 24 h at 37°C , 100 rpm. After cooling to room temperature, 200 µL aliquots of each solution were reacted with ninhydrin-citric acid and the absorbance read at 600 nm on a Cary-50 UV-Vis spectrophotometer (Agilent, Santa Clara CA). Susceptibility to collagenase is reported in terms of mmoles of amino acid released per mg of collagen as compared with a standard curve for leucine. Effectiveness of a tanning agent to protect against collagenase digestion is estimated by comparing the available amino groups after collagenase treatment with those available without collagenase treatment.

RESULTS AND DISCUSSION

Spectrophotometric Characterization of Soluble Collagen

A positive band near 223 nm and a stronger negative band at 198 nm characterize the circular dichroism (CD) spectra of native triple helical collagen in dilute solution at pH 3 - 4 and temperatures below 20°C . Figure 1a shows the CD spectra at 10°C before and after a 10°C to 60°C melting experiment of soluble collagen at 0.75 mg/ml in acetate buffer at pH 4. Under these conditions, the lower wavelength band is somewhat diminished, and red shifted to 203 nm, primarily due to the absorbance of light by the acetate ions. Despite being cooled to 10°C after the melting experiment, only 10% of the helical structure was recovered.

The apparent melting curve obtained by recording the CD signal at 223 nm as a function of temperature between 10°C and 60°C gives an indication of the stability of the helical

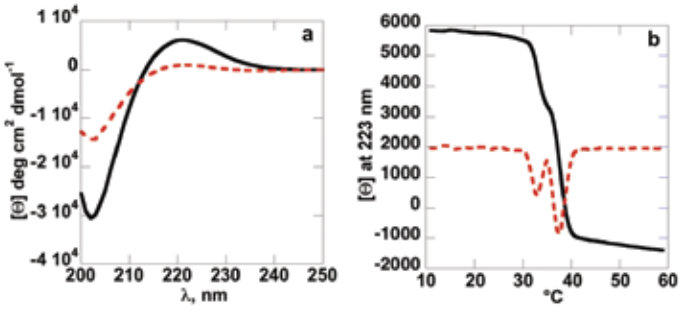

Figure 1. Soluble collagen at pH 4; (a) CD spectrum at 10°C before (——) and after (- - -) melting. (b) Molar ellipticity (——) at 223 nm recorded every 0.5°C over the 10°C to 60°C range with a time constant of 10 sec and a 7 deg/h heating rate and first derivative plot (- - -) of the melting curve.

conformation in collagen. In an early study[4] we attempted to monitor both the 223 nm and 198 nm bands and to use the absolute value of the difference in magnitude between them as a measure of the percent helical structure. However, the CD signal in the far-UV is less reliable due to higher noise levels. In the present study we focus on the 223 nm band where noise levels are inherently lower, acetate absorbance is less of a problem, and higher concentrations of collagen can be studied.

The helix-to-coil transition of bovine skin collagen in slightly acidic solutions (pH 3 - 5) is characterized as a two-step process, a predenaturation transition (T1) followed by complete denaturation (T2). The two-step nature of the melting is clearly seen in the melting profile (Figure 1b). The T1 and T2 temperatures were obtained from the derivative of the melting curve.

Recent studies of interactions of tannins with powdered hide were performed in phosphate buffered saline (PBS) at pH 6.[12, 13] When experiments using soluble collagen in PBS were attempted, the collagen was less soluble in PBS than in more acidic solvents, and the melting curve yielded a single

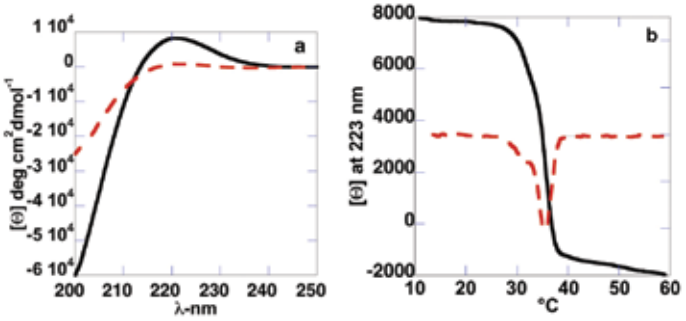

Figure 2. Soluble collagen treated with citrate masked $Al_2(SO_4)_3$; (a) CD spectrum at 10°C before (——) and after (- - -) melting. (b) Molar ellipticity (——) at 223 nm recorded every 0.5°C over the 10°C to 60°C range with a time constant of 10 sec and a 7 deg/h heating rate and first derivative plot (- - -) of the melting curve.

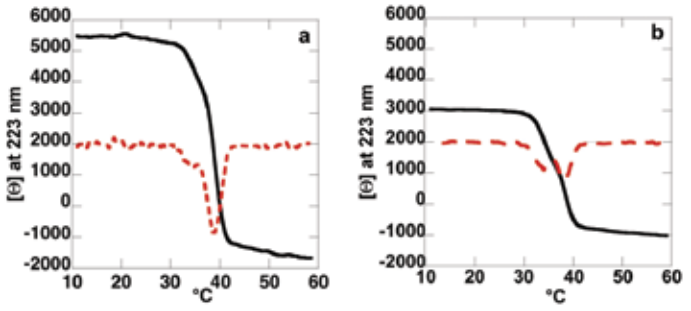

Figure 3. Soluble collagen treated with (a) chestnut tannin (b) quebracho. Ellipticity (——) at 223 nm recorded every 0.5°C over the 10°C to 60°C range with a time constant of 10 sec and a 7 deg/h heating rate and first derivative plot (- - -) of the melting curve.

transition at ~35°C . Treatment of collagen in PBS with the condensed tannin, quebracho, caused most of the collagen to aggregate and form a gel. The single transition temperature for the 5% of collagen remaining in solution was ~50°C . Thus simulated tanning of soluble collagen in PBS was feasible, but following the reaction in solution, proved impractical.

Al^{III} in an aged or masking solution is often the mineral of choice for combination tannages. The effects on the melting profile of soluble collagen of a freshly prepared $Al_2(SO_4)_3$ solution, a 24 h aged aluminum solution, and a citrate masked aluminum solution were compared. Aluminum sulfate solutions are colorless, a major plus for spectrophotometry, but their acidic nature (~pH 2) likely affects the stability of the collagen helix. The effect of aluminum sulfate on the wavelength scans was a slight blue shift (Figure 2a). Both freshly prepared and aged aluminum sulfate lowered T1 and T2 by ~ 2°C each, with the T1 peak to becoming a shoulder on the T2 peak (Figure 2b). Citrate masked aluminum sulfate lowered these values by 3 degrees each, and caused a decrease in the size of the T1 transition relative to that of the T2 transition. One concern when describing the interaction of Al^{III} with collagen is the probability that the tanning solution contains several different Al^{III} species. Reported structures include mononuclear complexes, linear 2 - 4 Al^{III} species and Al^{III}_{13} Keggin-like structures.[14] There is currently no method for distinguishing the interactions of individual aluminum species with collagen.

Reaction of tannins with soluble collagen resulted in the deposition of a colored gel on the protein side of the dialysis membrane. This gel formation removed collagen from the solution, thus lowering the concentration of soluble collagen available for the CD study, and because the far-UV absorbance spectra of collagen and tannin overlap, collagen concentrations could not be accurately determined. Nevertheless, because the tannins do not have a melting profile, in this temperature range, they did not interfere in the determination of transition temperatures, T1 and T2, for the collagen. The addition of chestnut (hydrolyzable) tannin to the collagen solution resulted in a diminishing of the T1 transition (Figure 3a) so that it became a shoulder on the T2 transition. It appears that the tannin may remove more of the collagen fraction that contributes to T1 from the solution. The addition of quebracho

TABLE I
Thermal transitions in collagen.

Collagen (C)	T1	T2	n
C in PBS	35.9 ± 2.2	43.9 ± 3.5	5
C at pH 4	32.5 ± 1.5	39.1 ± 3.6	10
C + Al	31.6 ± 1.8	36.2 ± 4.5	6
C + Al citrate	29.8 ± 1.1	33.6 ± 1.1	2
C + Q	35.1 ± 3.3	42.5 ± 7.9	6
C + Al – Q	32.5 ± 0.5	ND	3
C + Q – Al	gel	ND	3
C + AlQ mix	32.1 ± 0.6	35.5 sh	3
C + CH	34.4 ± 0.6	38.6 ± 0.4	6
C + CH – Al	33.7 ± 0.3	ND	3
C + Al – CH	33.7	ND	1
C + AlCH mix	31.6	35.5	2

Abbreviations: C – collagen, PBS – phosphate buffered saline, Q – quebracho, CH – chestnut, ND – not detected, n – number of determinations, sh – shoulder.

(condensed) tannin to the collagen solution removed more of the collagen from solution, depositing a brownish collagen/tannin gel on the membrane. The apparent helical content at 10°C is about 50% of what would be expected for the starting collagen concentration. Nonetheless, the shapes of the melting curve and its derivative (Figure 3b) are consistent with those for native collagen, suggesting that protein concentration may be the major difference.

We have examined the effects, on structure and stability of the remaining soluble collagen, by the three primary paths to an aluminum/tannin combination tannage. These paths are application of a mixture of aluminum with tannin, aluminum followed by tannin, and tannin followed by aluminum. The resulting T1 and T2 values are summarized in Table I. Mixtures of either chestnut or quebracho tannin with aluminum had little effect on T1, and lowered T2 by 3 to 4°C. The treatment of collagen with aluminum followed by tannin resulted in a single transition at a temperature near T1 for native collagen. A general principle in the use of equilibrium dialysis cells is that the cavities on either side of the membrane are filled to the same level, and the levels should remain the same throughout the experiment. This principle held, except when the treatment was with tannin followed by aluminum. When the collagen/tannin complex was on one side of the membrane, and the aluminum solution on the other side, the liquid level on the aluminum side began to rise as tannin solution was extracted across the membrane. Ultimately, there was a collagen/tannin gel on one side, and a tannin/aluminum mixture on the other. In the case of quebracho, gelation was complete, with no dissolved collagen remaining. With chestnut, the extraction was less complete leaving a small amount of soluble collagen/chestnut complex in solution, a result consistent with observations of Hagerman *et al*.[15] that condensed tannins are somewhat more effective than hydrolyzable tannins at precipitating proteins. In addition, these results confirm the conclusions of earlier researchers that aluminum reacted more strongly with tannins than with collagen.[3, 16] Whether the predenaturation transition (T1) is due to minor defibrillation of the collagen in solution,[17, 18] or to the existence of hydroxyproline deficient, thermally labile domains in collagen[19] is still a topic for debate. The effects seen in this study of aluminum and tannin either alone or in sequence on the melting profile are more consistent with a defibrillation that makes more interaction sites available, than with a labile domain. The lack of effect of aluminum-tannin mixtures on the melting profile of collagen is good evidence of the stronger attraction of aluminum for tannins than for collagen.

Electrophoresis

Electrophoresis patterns (not shown, data summarized in Table II) for the untreated collagen were similar to those reported by Lin and Liu,[20] with major bands for the collagen b-chain near 200 kDa, and the α1- and α2- chains slightly above the 116 kDa marker. A small fraction of the material in each sample was in large aggregates that did not penetrate the separating gel, but remained above it in the lower density stacking gel. Electrophoresis patterns for soluble collagen treated with either hydrolyzable or condensed vegetable tannins were essentially the same as those for untreated collagen, in agreement with results from our previous study of polyphenol-modified gelatin.[21] In contrast, patterns for soluble collagen treated with aluminum sulfate, in any of the forms used here, alone or with vegetable tannins showed only high molecular weight material that did not penetrate the gel.

TABLE II
Electrophoretic patterns.

Sample	Pattern	N
Collagen (C)	collagen	1
C + Tan	collagen	5
C + Al	high MW	4
C + Al/Tan mix	high MW	3
C + Al — Tan	high MW	3
C + Tan — Al	high MW	3

See Table I for abbreviations, Tan – either CH or Q, collagen pattern consists of 3 bands between 150 and 220 kD, high MW - no sample moved from the stacking gel into the separating gel.

TABLE III
Collagenase resistance.

Collagen	w/wo	N
Collagen (C)	2.5 ± 0.6	1
C + Al	1.5 ± 1.0	4
C + CH	0.9 ± 1.0	3
C + Al — CH	2.2 ± 1.5	3
C + CH — Al	0.01	3

w/wo – ratio of available primary amino groups after treatment with collagenase to without collagenase

Collagenase Resistance

Collagenases are a class of enzymes that cleave the triple helical structure of collagen. Treatment of collagen with collagenase increases the number of available primary amino groups by exposing epsilon-amino groups on previously buried lysine residues, as well as by creating additional N-terminal amino groups. Tanning which protects the hide from putrefaction, would be expected to enhance its collagenase resistance. Under the digestion conditions used in this study (results summarized in Table III) the number of available primary amino groups after collagenase digestion of untreated soluble collagen, was about 2.5 times what was available without digestion, in agreement with earlier findings.[22] For aluminum treated collagen, the increase was less, about 1.5 times, showing some stabilization. For collagen treated with chestnut tannin, the collagenase reaction yielded no increase in available amino groups, in other words, the collagen was fully protected by the tannin. Collagenase produced nearly the same increase in available amino groups when the collagen had been treated first with aluminum and then with chestnut as when there was no treatment. This result suggests that the interaction between aluminum and chestnut was stronger than that between aluminum and collagen. In contrast, the interaction of collagen with chestnut, followed by aluminum, resulted in a more stable collagenase resistant complex that formed a gel on the membrane as water was extracted from the protein-containing cavity into the aluminum solution.

CONCLUSIONS

This study, by focusing on the effects of tanning agents on soluble collagen, rather than on intact or powdered hide, gives a new perspective on the tanning process. The results confirm the more effective protein precipitating power of condensed over hydrolyzable tannin, and the greater affinity of aluminum for tannin than for the collagen helix. The importance of the aluminum/tannin reaction is clear, and the extraction of water from the collagen solution in the formation of the aluminum/tannin complex suggests that the lowering of water activity around the collagen may play a role in stabilizing a collagen-tannin-aluminum tannage. An interesting finding is that aluminum, which has little effect on collagen helical structure and stability, does appear to connect collagen molecules in some manner to produce high molecular weight species that do not separate under the conditions of SDS PAGE. On the other hand, the reaction of tannin with collagen did not alter the molecular weight profile of collagen.

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