

Model Systems for Leather Research and Beyond

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Abstract

Animal hides and skins, the most valuable byproducts of the meat industry, are raw material for the leather, biomaterials, gelatin and glue industries. Each of these industries modifies its processing methods as concerns over safety, the environment or economics arise. Processing changes are generally evaluated in terms of impact on quality of product and costs to the industry, with little regard for the effects on downstream industries. Because the basis for tanning and other biomaterial applications is the stabilization of the collagen matrix, changes to the molecular characteristics of hide collagen may be expected to impact these applications. We have begun the development of protocols using hide samples removed from different stages in the beamhouse processes to evaluate the effects of processing changes. For example, a variety of dehairing processes are currently in use or under development. Do these different processes affect the tanner's or the biomaterials engineer's or the gelatin manufacturer's substrate? Our model systems use intact and powdered hide and extracted collagen as well as our computational model. The results are anticipated to assist the tanner as well as the manufacturers of collagen-based biomaterials and gelatin in better understanding their substrate and changes to it that may occur when beam-house processes are altered.

Keywords: molecular modeling, soluble collagen insoluble collagen, collagen stabilization, crosslinking

Introduction

Leather production is a byproduct industry, without which the hides of slaughtered animals would become a major disposal problem for the meat industry. The conversion of animal skins and hides into leather was one of humankind's early activities. Over millennia, several classes of tanning agents with a variety of properties were identified. The history of each of the three broad categories (mineral, vegetable, aldehyde) of tannages in use today can be traced at least to 2000 BC. From a practical perspective, each tanning process is a multistep operation that stabilizes the collagen matrix of the hide, giving it strength and resistance to organisms that would otherwise attack and destroy the hide. Converting a skin or hide into leather is always a macro scale process even for the smallest tanning operations. For most of history, tanning processes were developed as arts or crafts because the science behind tanning was largely inaccessible. In the late 19th and early 20th centuries, leather science became an academic specialty that attracted many noted scientists to the study of collagen as related to leather.

Fibrous collagen, the main protein of the extracellular matrix, gives strength and form to the skin of mammals and serves as the substrate for the production of leather and numerous biomaterials for other industries, including medical materials, food and adhesives. The insolubility and noncrystalline nature of

the collagen presented a challenge to scientists who attempted to elucidate its structure for physiological or technological purposes. This challenge was addressed by development of model systems for the study of tanning reactions. By mid 20th century, model systems based on soluble collagen (gelatin) and insoluble collagen (hide powder) were in common use for studies of the interactions of each of the categories of tanning agents with collagen (Gustavson 1949), and molecular models of collagen structure were beginning to be developed by Ramachandran and Kartha (1954).

Scientists at ERRC, USDA have long performed research in the area between the basic science of the academy and the applied technology of an industrial setting, with the aim of balancing basic and applied approaches to each research problem. One of the early steps in most projects has been the design of a model system with which to explore a hypothesis. Early models were likely to be sheep or calfskin to limit the amounts of chemicals needed. Much of the knowledge developed in this manner has ultimately been incorporated into industrial processes. Scientific advances of the twentieth century, including increasing use of collagen in medical biomaterial research, began to provide a basis for understanding the relationship between collagen structure and function in both biology and technology.

In recent years, consumer preference and environmental regulation, have driven research into the development of alternative methods for processing hides. An understanding of the mechanisms of hide preparation and tanning will provide a scientific basis for the design of economical, environmentally friendly processes to produce high quality leathers. Broadly speaking, there are three categories of model systems with which to study collagen in the context of tanning, food uses, and biomaterials. Soluble models now generally employ pepsin treated collagen to study the effects of isolated tanning reactions on the conformation and conformational stability of collagen in solution. Solid models may be purified collagen or powdered hide isolated from various points in processing. Molecular models allow one to simulate tanning processes. Since the 1989, scientists at ERRC, USDA have contributed to the development of each of these categories of models. This paper will briefly describe these contributions, and suggest how they may be applied to studies of collagen stabilization in general and to tanning in particular.

Computational model

In 1989, using conformational parameters developed by the Scheraga group (Miller et al. 1980), the then available amino acid sequence of type I collagen (Fietzek and Kuehn 1976), X-ray diffraction data (Eikenberry and Brodsky 1980), and a proposed five-helix microfibril structure (Smith 1968) we began the construction of a 36 residue long collagen model capable of visualizing interactions between helices Chen et al. (1991 a,b,c). Over the next several years, procedures were developed for lengthening the model to represent a complete D-space, and for replacing proline and hydroxyproline in the collagen template with correct amino acid sidechains from the sequence without disrupting the backbone structure King et al. (1996). Because the individual chains were staggered, the entire tropocollagen sequence was represented. Initially, the nonhelical telopeptides that serve as anchors for native collagen crosslinks were ignored because of the limited data then available on their secondary structures. By 1999, available secondary structures for isolated telopeptides encouraged us to construct models of N- and C-terminal telopeptides (Qi and Brown 2002; Brown 2004) and to attempt to fit them into the larger model.

This model has recently been updated with amino acid sequence data for the bovine $\alpha 1(I)$ chain (GenBank ID: AAI05185.1) and $\alpha 2(I)$ chain (NCBI ID: NP_776945.1; Zimin et al. 2009), and

conformations for the N- and C- terminal telopeptides as described by Malone and co-workers (2004 a,b). The updated model represents a segment of a cylindrical assemblage of five triple helices such that all parts of the bovine type I collagen sequence can be visualized in relation to neighboring helices. A description of the development of this model has been published, with the complete set of coordinates, as supplementary material Brown (2013).

The impetus for construction of this model was to provide a basis for the study of interactions between small molecules and collagen that might be expected to contribute to the stabilization of the fibril structure. For crosslinking reactions, we have used this model to explain the effectiveness of C-7 to C-12 dicarboxylic acids as stabilizers on the basis of the potential to form crosslinks that span helices. For the chrome tanning process, if one can assume that the formation of crosslinks between bi- or trinuclear Cr(III) sulfate complexes and carboxyl group side chains on collagen are a part of the mechanism, a carboxyl to carboxyl distance, on different helices, in the 0.6 to 0.8 nm range would be required (Gotsis et al. 1992). The number of carboxyl groups available for the formation of productive crosslinks is in excellent agreement with early estimates based on experimental data combined with an alignment of the collagen sequence to produce triple helices with hierarchical structures (Covington 1997). Genipin, a naturally occurring biocompatible crosslinking agent that self polymerizes, is predicted to bridge peptide chains at distances of 1.6 to 2.5 nm (Liang et al. 2004). This collagen microfibril model provides a basis for estimating the contributions of genipin bridges of different lengths, and the potential for a maximum of 22 genipin bridges (Brown 2013).

To explore the relative importance of hydrophobic interactions or hydrogen bonding in vegetable tanning, model tannin molecules were docked into positions near hydroxyl groups on serine residues, or hydrophobic sidechains and allowed to move freely under molecular dynamic simulations, at virtual temperatures up to 400 K, while the microfibril was restrained (Figure 1). Changes in distance between the tannin molecule and selected nearby sidechains under the simulation suggested that hydrophobic interactions were at least as important as hydrogen bond formation (Brown et al. 1997).

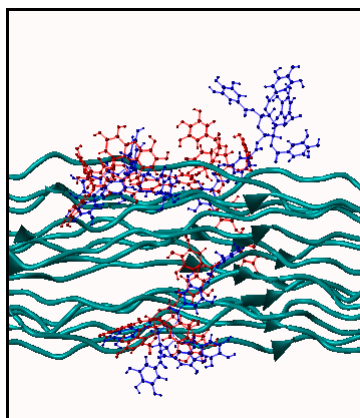


Figure 1. A short segment of the microfibril model (cyan ribbon) with three gallotannin molecules (blue) before molecular dynamics simulation and after simulation at 400K (red) while the alpha carbons of collagen were immobilized.

Water is an important factor in the stabilization of the collagen matrix, and models that explore the interactions of bound and free water on collagen peptides are common (Madhan et al. 2003; Zhang et al. 2007; De Simone et al. 2008). Although the computational expense of including explicit water in the full microfibril model is significant, studies using explicit solvent systems on shorter microfibril models to simulate effects on collagen fibers have been reported (Bronco et al. 2004; Siggel and Molnar 2006; Buló et al. 2007). Under molecular dynamics simulations, the microfibril conformation was shown to be less stable in water than in dilute solutions of formaldehyde or gallic acid/water (Bronco et al. 2004). The effects on collagen swelling with pH changes in different salt solutions, an aspect of leather processing, were reproduced (Bronco et al. 2004; Siggel and Molnar 2006; Buló et al. 2007).

For an exploration of the role of water in the interactions of vegetable tannins with collagen, fragments of the overlap and gap regions of the microfibril model were excised, then energy minimized gallotannin models were docked into energetically favorable positions on each fragment. The α -carbon backbone of collagen was kept immobile during molecular dynamics simulations at virtual temperatures from 400 K to 800 K, with and without an added layer of water, to identify possibly more favorable interaction sites for the gallotannin molecules. Both inter- and intra-chain interactions were identified, along with potential sites for hydrogen bonding or hydrophobic interactions (Brown and Shelly 2011).

Computer-assisted molecular modeling is a tool for visualizing structure-function relationships in proteins, and for predicting the effects of proposed modifications to protein structure. This model was developed for exploring mechanisms for interactions of tanning chemicals with collagen, and has proved useful in the analysis of crosslinking reactions. It is anticipated that the designers of collagen-based biomaterials including new tannages for leather may find it useful.

Soluble collagen model

To complement computer modeling studies, we developed laboratory scale model-tanning systems. For a soluble collagen model, pepsin-solubilized collagen was characterized by SDS-PAGE and ultraviolet and circular dichroism spectroscopy to establish that it was an acceptable model for the native characteristics and thermal stability of triple helical collagen. It was then used as a soluble model to explore the interactions of chromium (III)- and aluminum sulfate complexes with collagen in solution (Brown et al. 1997; Brown and Dudley, 2008). Measurable effects on the conformation and conformational stability of soluble collagen under the conditions of tanning could be observed. Among our observations was that even in solution effective chromium fixation occurred only with a slowly rising pH. Investigation of the effects of added neutral salts on the thermal stability of the collagen triple helix suggested that salt levels typical of tanning processes would have water-structuring effects around the collagen molecules (Brown et al. 2000). Studies of vegetable tannins with soluble collagen are currently underway and are expected to identify specific reactions in the vegetable tanning process. Advantages of the soluble collagen model are that reactions may be studied at the molecular level using the typical techniques of protein physical chemistry including spectrophotometry and gel electrophoresis.

Insoluble collagen models - fibrils

Collagen fibers isolated from bovine splits were ball-milled to separate fibrils with diameters in the 50 - 100 nm range from the larger fibers. During milling, the collagen was characterized with respect to active surface area per mass, fibril structure and molecular weight distribution. Active surface area, defined as the surface area per gram able to interact with the liquid in which the collagen was blended, increased from $\sim 0.1 \text{ m}^2/\text{g}$ to $\sim 30 \text{ m}^2/\text{g}$ during the milling process, exposing additional reaction sites for either tanning or other stabilizing materials (Maffia et al. 2004). These fibrils could be further unraveled when treated with low frequency, high power ultrasound (20 kHz) without degrading the collagen (Brown et al. 2006). The stabilizing or crosslinking effectiveness of glutaraldehyde, microbial transglutaminase and dehydrothermal drying on these fibrils were evaluated in terms of changes in number of potential binding sites, molecular weight distribution and resistance to collagenase degradation. Treatment with glutaraldehyde, a component of many wet white tannages, resulted in the least number of free amino groups (binding sites) remaining, the highest molecular weight aggregates and greatest resistance to collagenase degradation. Transglutaminase, while not effective as a tanning agent in the bovine hide matrix, is widely used by the food and pharmaceutical industries to stabilize collagen-based biomaterials. Treatment of fibrils with transglutaminase produced high molecular weight aggregates that were more resistant to collagenase than control collagen. More free amino groups remained after transglutaminase treatment, which uses one amino group per crosslink, than after glutaraldehyde treatment that uses two amino groups per crosslink. Dehydrothermal drying, not important for tanning, but favored by some biomaterials engineers, produced high molecular weight aggregates that were more susceptible to collagenase than control materials (Lastowka et al. 2005).

Insoluble collagen models - hide powder/powdered hide model

A second laboratory scale insoluble collagen model uses pulverized bovine hide, commercially available as "hide powder," a proprietary material intended for the analysis of vegetable tanning agents or enzyme activity. Pulverized hide is the model most representative of intact hide and has the advantage that with good sampling procedures a few grams of powdered hide can be more representative of the hide than an intact piece of similar weight cut from the hide. To evaluate the effects of changing beamhouse processes on characteristics and quality of collagen as a substrate for tanning or a byproduct for sale to other industries, we have developed a generic protocol for preparation of powdered hide from different points in the hide preparation/tanning process. Powdered hide from different processes or at different stages in a process was characterized in terms of moisture, ash, total protein as collagen, hydrothermal stability, collagenase resistance, proteoglycan content and molecular weight distribution (Brown et al. 2010).

Environmental and economic issues may dictate changes in beam-house processes to prepare the hide for tanning. New processes may be implemented without a full understanding of their effects on the chemical and physical properties of the resulting leather, or on the collagen byproduct the tannery provides to the biomaterials industries. Removal of hair from the hide is an area where processes that use distinctly different chemistries are being evaluated. Multiple variations of

sulfide, oxidative and enzymatic dehairing processes are currently in use worldwide. Because the effects of different processes on the molecular characteristics of collagen have thus far received little attention, we prepared powdered hide from sulfide dehairied hide and an oxidatively dehairied hide for a comparative study. In this particular case, the characteristics of extractable collagen from the two hides were remarkably similar while there were distinct effects on the collagen fiber structure (Brown et al. 2012). Fiber bundles in the oxidatively dehairied powdered hide were large (2 – 5 μm) with discernable individual strands in the 0.5 – 0.8 μm range while fiber bundles in the sulfide dehairied powdered hide were more compact with fewer distinguishable strands (Figure 2).

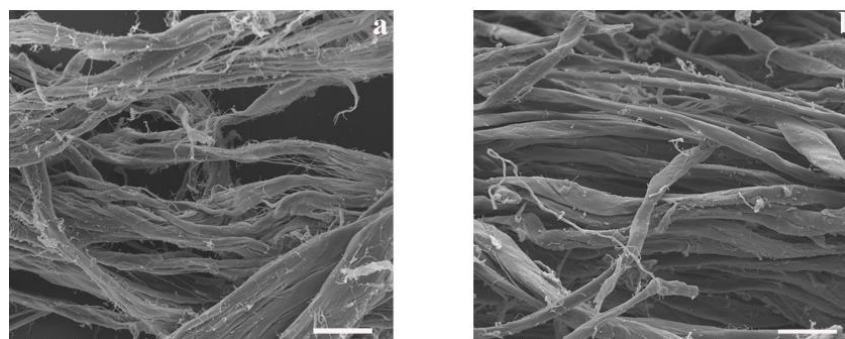


Figure 2. Scanning electron micrographs of powdered hide, 1000x magnification, bar represents 20 μm . Samples are (a) powdered hide from oxidative dehairing and (b) powdered hide from sulfide dehairing.

When powdered hide samples prepared from individual steps in the beamhouse process were compared, the similarities in properties between the powdered hide from sulfide and oxidative dehairing were more notable than the differences. Differences decreased as the hide was processed through to the wet blue stage (Brown et al. 2013).

The powdered hide model provides leather chemists and technologists a relatively standard protocol for research on tanning mechanisms at an intermediate level between soluble collagen and intact hides, and will facilitate collaborations and information exchanges among laboratories around the world.

Conclusions

Tanning processes continually evolve in response to environmental, economic and legislative pressures. As these processes evolve, there may be unanticipated effects on the final leather or on the byproducts that are intended for use by the food and biomaterials industries. The use of model systems can contribute to an understanding of current processes and the development of effective sustainable tanning processes to produce leathers with desirable physical and subjective properties. The model systems described here provide tools for evaluating the effects of tanning and pre-tanning steps. This research provides a basis for assessing the effects of different process steps in a processing system and represents an initial step in the development of well-characterized procedures for comparing research from different laboratories on tanning mechanisms. The results obtained from multiple models are anticipated to assist the tanner as

well as the manufactures of collagen-based biomaterials and gelatin to better understand their substrate and changes to it that may occur as processes evolve.

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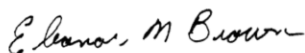
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