# Powdered Hide Model for Vegetable Tanning II: Hydrolyzable Tannin

*by*

Eleanor M. Brown,* Maryann M. Taylor and Lorelie Bumanlag

*U.S. Department of Agriculture, Agricultural Research Service*

*Eastern Regional Research Center***

600 East Mermaid Lane

Wyndmoor, PA 19038

## Abstract

Vegetable tannages employ both condensed and hydrolyzable tannins. As part of our exploration of tanning mechanisms, we reported previously on interactions of the condensed tannin, quebracho, with powdered hide. In this study, the interactions of chestnut extract, a hydrolyzable tannin, with powdered hide samples are reported and compared with those of the condensed tannin. Hydrothermal stability of powdered hide treated with the hydrolyzable tannin reached a maximum of 75° C at a 40% offer, compared with 84° C for a similar offer of condensed tannin. The hydrolyzable tannin was much more effective at improving collagenase resistance, with nearly complete protection at <10% offer.

## Introduction

Tanning, the conversion of animal hides into leather can be accomplished by several distinct processes. The three major classes of tanning reagents (metals, polyphenols, and aldehydes) each produce a material with distinctive characteristics that is clearly leather. Chrome-tanned wet blue, aldehyde-crosslinked wet white, and vegetable tanned hides are major contributors to current leather production. The choice of specific reagents within each class tends to evolve over time without a thorough understanding of the relevant tanning mechanisms. Tanners also adjust their processes in response to new regulations from their governing bodies or the availability of new formulations from suppliers. As new reagents and processes are adopted, the effects of individual steps in the process on the hide substance are poorly understood. A seemingly minor change in one part of a beam-house process may lead to additional changes in later stages.

Vegetable tanning, the oldest of the currently used tanning technologies is, from a mechanistic perspective, the least well understood. Some combination of hydrophobic interactions and hydrogen bonds between polyphenolic vegetable tannins and collagen[1] are likely, as is a filling action in the gap region of the collagen fiber.[2] Both condensed tannins, based on a heterocyclic ring system, and hydrolyzable tannins containing a central sugar moeity[3] are used in vegetable tanning. In this study, we use the powdered hide model to begin to explore possible vegetable tanning mechanisms, to compare a hydrolyzable tannin with a condensed tannin, and to assess the effects of oxidative and sulfide dehairing in vegetable tanning.

*Corresponding author e-mail address: eleanor.brown@ars.usda.gov

Presented in part at the 110th annual American Leather Chemists Association Meeting, June 18-20, 2014 Saratoga Springs, NY

**Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

The primary purpose of tanning is to stabilize the collagen matrix of the hide or skin so that it has improved hydrothermal stability and is more resistant to microbial attack. Our approach has been to develop model systems, experimental models at the bench and pilot plant level, and computational models for evaluating the effects of beamhouse processes on collagen. The current laboratory model[4] uses powdered hide prepared from hide that was dehaired by a traditional sulfide process[5] or by an oxidative process.[6] We have used our powdered hide model system to show that there is little difference in the behavior of sulfide and oxidatively dehaired hides through the chrome tanning process.[4,7] More recently, we adapted the model for examining the interactions of quebracho, a condensed tannin with collagen.[8] In this study we look at the hydrolyzable tannin extracted from chestnut bark. The collagen microfibril model[9] that showed preferences for hydrogen bonding and hydrophobic interactions in the interactions of condensed tannin with the collagen microfibril[1,8] will be used to explore interactions of hydrolyzable tannin with collagen.

## EXPERIMENTAL

### Materials

Bacterial collagenase (361 units/mg) from *Clostridium histolyticum*, and pepsin from gastric mucosa, were obtained from Sigma-Aldrich, St. Louis. Tannins, quebracho and chestnut, were obtained from Hermann Oak Leather Company (St. Louis, MO). All other chemicals were reagent grade from various suppliers.

### Sample Preparation

#### *Tannin Preparation*

Commercially available tannins were extracted with hexane to remove lipids, chlorophyll, and other nontannins[10,11] and dried under vacuum. Concentrations of soluble tannin were estimated from the UV absorbance at 280 nm of a solution in phosphate buffered saline (PBS). Absorbances of 20.9 for chestnut, and 18.6 for quebracho on a mg/ml basis were estimated from the spectra of replicate experiments.

#### *Powdered Hide Preparation*

Powdered hide from the delime/bate step of beamhouse processing was prepared as previously described.[4] Two batches of powdered hide were used in this study, one from hide that was sulfide dehaired and relimed with lime/sulfide as described by Cabeza et al.[5] and the other from hide that was oxidatively dehaired and relimed without sulfide as described by Marmer and Dudley.[6]

#### *Tanning of Powdered Hide*

Sulfide (S-PH) and oxidatively dehaired powdered hide (Ox-PH) samples 400 to 1000 mg were hydrated in 15 ml of phosphate buffered saline (PBS) at pH 6 or pH 2, containing various amounts of tannin. The reaction was run overnight, either at room temperature with stirring, or in an incubator at 35°C with rotation at 90 rpm. Samples from reactions at pH 2 were adjusted to pH 6 with 1% sodium bicarbonate. Tanned powdered hide suspensions were filtered with gentle vacuum on Whatman #1 paper. The volume of the filtrate was measured and a portion reserved for analysis. The filter cake was then washed with 100 ml water to remove excess salt and unbound tannin. The concentrations of tannin in the filtrate and wash were estimated from the absorbance at 280 nm.

### Characterization of Tanned Powdered Hide

The wet filter cake was subdivided into samples for moisture, thermal stability of the tanned powdered hide, and stability of collagen structure. For moisture determination, ~120 mg of wet filter cake was weighed into a dried weighing bottle. The bottle with wet filter cake was placed in a vacuum oven with vacuum but no heat for 24 h, and then reweighed.

#### *Calorimetry*

Hydrothermal stability of tanned powdered hide was determined on a Multi-Cell Differential Scanning Calorimeter (DSC) (model CSC-4100) from Calorimetry Sciences Corporation, Lindon, UT, as previously described.[4] Moist, blotted samples (~200 mg dry weight) were weighed into ampoules, and dispersed in 100mL water to assure good contact with the heating element. The filled ampoules were sealed and placed in the calorimeter which was programmed to record heat flow as mcal/°C while the temperature was increased from 30°C to 130°C at 1.0°C/min with an equilibration period of 600 s at the start. The temperature at the peak of the calorimetry trace, $T_{p,}$ was considered to be an apparent shrinkage temperature.

#### *Collagen Stability*

Tanned and untanned powdered hide was extracted first with 0.5 M acetic acid and then with the addition of pepsin as described previously[4] to determine the extractability of collagen. Collagenase susceptibility of powdered hide from the sulfide and oxidatively dehaired hide at the delime/bate step and after tanning with chestnut or quebracho tannin was analyzed as described in earlier studies[12] with modification of the detection method to use water rather than propanol for the dilution, as reported by Zhang et al.[13]

#### *Computational Model*

A 756-residue segment was excised from the gap region of the microfibril model.[9] This segment contained 12 chains, each 63 residues long, in the form of 4 triple helices. ACE and NME caps were added to the amino and carboxyl termini of each chain. The model for condensed tannins was taken from previous studies[1,8] and a model hydrolyzable tannin, β–pentagalloylglucose was constructed as described by Vivas et al.[14] In separate experiments, each of these models was merged into the same relatively open position in the microfibril. After the positioning of the tannin molecule in the microfibril was optimized by energy minimization, the capped ends of the chains were anchored to prevent disintegration of the protein during dynamics simulation. The molecular complex was then subjected to molecular dynamics

simulations at 300K for 10 ps. The movement of the tannin molecule relative to selected residues in the microfibril was monitored as a function of time.

## RESULTS AND DISCUSSION

### Uptake of Tannin by Powdered Hide

The relationship between offer and uptake of hydrolyzable (chestnut) tannin and condensed (quebracho) tannin was relatively linear and nearly the same for powdered hide from either a sulfide or an oxidative dehairing process (Figure 1). Uptake from offers in the 20-40% range was consistently lower than anticipated, but being outside the typical offers used in tanning operations, probably not significant.

### Calorimetry

Table I details the apparent shrinkage temperatures obtained by DSC with powdered samples treated with varying offers of chestnut or quebracho tannin.

The apparent shrinkage temperature increased linearly as the offer of chestnut was increased up to 40% on either sulfide or oxidatively dehaired powdered hide, and then remained constant. In this study, the thermal stability of quebracho-treated powdered hide was constant above an offer of 30%. These results suggest that the rationale for using very high offers of tannins[15] is to assure an uptake of at least 40%. Hydrothermal stability of quebracho treated powdered hide was nearly 10°C higher than the chestnut treated material.

### Stability of Collagen Structure

Extraction of 0.5 g of chestnut treated S-PH and Ox-PH with 0.5 M acetic acid did not yield measureable amounts of collagen. The addition of pepsin in the acetic acid extraction was equally ineffective at liberating significant collagen from these chestnut treated samples.

### Susceptibility to Collagenase

Collagenases are a class of enzymes that cleave the triple helical structure of collagen, and increase the number of available primary amino groups by exposing buried sidechain groups and creating additional N-terminal amino groups. Collagenase digestion using typical amounts of reagent and reaction time had no effect. When the sample size, amount of enzyme and length of time for digestion were all increased by nearly a factor of 10, the untanned S-PH was moderately susceptible with the liberation of 26 mμM/mg of amino groups, while untanned OX-PH was less susceptible (Figure 2). A control without enzyme was run with each sample so that any degradation resulting from the longer time of reaction could be noted. Chestnut tannin had a strong protective effect even at 19% offer. These results are consistent with the finding of Colak[16] that as little as 1% chestnut extract in the soaking float had excellent biocidal activity.

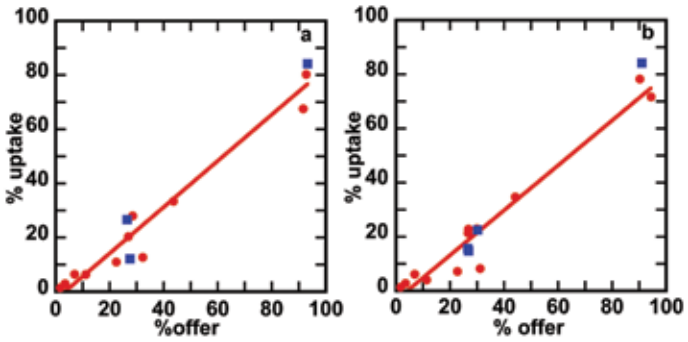


Figure 1. Uptake of chestnut (red) and quebracho (blue) by powdered hide from sulfide (a) or oxidatively dehaired hide (b).

**TABLE I**
**Apparent Shrinkage Temperature, °C.**

| Sample[a] | Sulfide dehaired | Oxidatively dehaired |
|---|---|---|
| 0% CH | 64.5 ± 3.2[b] | 66.3 ± 2.6 |
| 10% CH | 68.3 ± 0.8 | 67.8 ± 0.4 |
| 20% CH | 70.4 ± 0.5 | 69.5 ± 0.1 |
| 30% CH | 72.4 ± 0.6 | 71.0 ± 0.4 |
| 40% CH | 75.3 ± 0.3 | 74.7 |
| 90% CH | 74.3 ± 0.9 | 75.3 ± 0.3 |
| 25% Q | 82.7 ± 3.4 | 82.2 ± 1.3 |
| 90% Q | 82.8 ± 0.5 | 83.6 ± 0.5 |

[a]CH represents chestnut and Q represents quebracho.
[b]Values with ranges represent results obtained with at least two separate samples.

### Computational Model

In an earlier study, using the collagen microfibril model to explore interactions of model gallotannins with the collagen microfibril, both hydrogen bonding and hydrophobic interactions were observed.[1] At that time, tannin molecules were docked, near serine residues on the microfibril, and allowed to move freely relative to a static microfibril during molecular dynamics simulations at 300K - 400K. In this study, the tannins were merged into a relatively open area of the gap region and both tannin and collagen had a degree of motional freedom during the dynamics simulation. One notable difference between the tannin models is size, where the model condensed tannin molecule has 77% more atoms than the hydrolyzable tannin. During the dynamics simulation at 300K, the condensed tannin moved 0.29 nm relative to the microfibril while the hydrolyzable moved 0.59 nm, nearly twice as far; both moved toward areas where the number of potential H-bonds was greater. For the condensed tannin, the H-bond density was increased by 15% at the

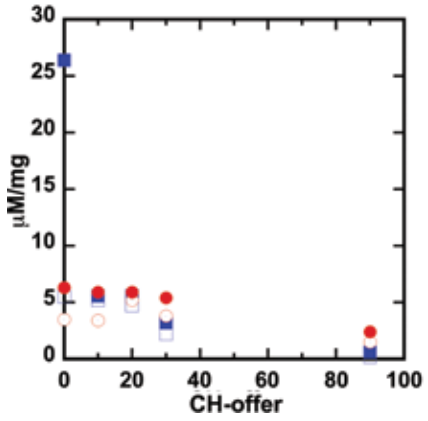


Figure 2. Available primary amino groups liberated from powdered hide as a function of chestnut offer and collagenase digestion. Solid markers represent amino groups liberated by collagenase, open markers are controls without collagenase. Blue markers represents results from sulfide dehaired hide and red markers represent oxidatively dehaired hide.

completion of the simulation, and for the hydrolyzable tannin the increase was 20%. Although both tannin molecules were initially positioned similarly within the microfibril, the difference in their size affected the apparent composition of the surroundings. The average number of residues within 0.6 nm of selected atoms in the tannin was initially 12 ± 1 for the condensed tannin model and 8 ± 3 for the hydrolyzable model. After dynamics simulation, the average for both types of tannin in this 0.6 nm sphere was 9 ± 2 residues. Based on the hydrophobicity values developed by Black and Mould[17] for post-translationally modified amino acid residues, there was a moderating effect where the surroundings of selected atoms in the condensed tannin became slightly less hydrophobic (0.44 ± 0.02, to 0.36 ± 0.1) while for the hydrolyzable tannin the effect was toward a slightly more hydrophobic surrounding (0.28 ± 0.02 to 0.36 ± 0.1).

## Conclusions

The tanning effect of a hydrolyzable tannin, chestnut, is most notable in protecting against microbial degradation. Even a very low offer protected the powdered hide from attack by collagenase. Hydrothermal stability reached a maximum of only 75°C at a 40% offer. Little difference was observed between the effects on S-PH and Ox-PH. The computational model shows that the smaller size of the hydrolyzable tannin allows it to move more freely within the microfibril than is possible for the condensed tannin. Presumably the greater mobility and ability to penetrate the hide substance allows it to protect more sites where collagenase could attack. The results of this study suggest that tanners and biomaterials engineers may want to consider the preservative qualities of very small amounts of hydrolyzable tannins in their formulations.

## Acknowledgements

The authors wish to thank Renee Latona for preparation of powdered hide, and Joe Lee for tanning advice.

## References

1. Brown, E. M. and Shelly, D. C.; Molecular modeling approach to vegetable tanning: preliminary results for gallotannin interactions with the collagen microfibril. *JALCA* **106**, 145-152, 2011.
2. Haslam, E.; Vegetable tannage: Where do the tannins go? *J. Soc. Leather Tech. Chem.* **81**, 45-51, 1997.
3. Hagerman, A. E.; Purification of quebracho tannin. In Tannin Chemistry http://www.users.muohio.edu/hagermae/ accessed May 2010.
4. Brown, E. M, Latona, R. J., and Taylor, M. M.; Powdered hide for research on tanning mechanisms. *JALCA* **105**, 116-120, 2010.
5. Cabeza, L. F., Taylor, M. M., DiMaio, G. L., Brown, E. M., Marmer, W. N., Carrio, R., Celma, P. J. and Cot, J.; Processing of leather waste: Pilot scale studies on chrome shavings. Part II. Purification of chrome cake and tanning trials. *JALCA* **93**, 83-98, 1998.
6. Marmer, W. N. and Dudley, R. L.; Oxidative dehairing by sodium percarbonate. *JALCA* **100**, 427-431, 2005.
7. Brown E. M., Latona, R. J., Taylor, M. M. and Garcia, R. A.; Effects of pretanning processes on bovine hide collagen structure. *JALCA* **107,** 1-7, 2012.
8. Brown, E. M., Latona, R. J., Taylor, M. M., Gehring, A. G.; Powdered hide model for vegetable tanning. *JALCA* **109,** 8-13, 2014.
9. Brown, E. M.; Development and utilization of a bovine type I collagen microfibril model. ***Int J Biol Macromol.*** **53**, 20-25, 2013.
10. Arapitsas, P.; Hydrolyzable tannin analysis in food. *Food Chemistry* **135,** 1708-1717, 2012.
11. Antoine, M.-L., Simon, C., Pizzi, A.; UV spectrophotometric method for polyphenolic tannin analysis. *J. Appl. Polym. Sci.* **91**, 2729-2732, 2004.
12. Brown, E. M., Stauffer, D. M., Cooke, P. and Maffia, G. J.; The effect of ultrasound on bovine hide collagen structure. *JALCA* **101**, 274-283, 2006.
13. Zhang Y., Fu, Y., Zhou, S., Kang, L., Li, C.; A straightforward ninhydrin-based method for collagenase activity and inhibitor screening of collagenase using spectrophotometry. *Anal. Biochem.* **437**, 46-48, 2013.
14. Vivas N., Laguerre, M., Glories, Y., Bourgeois, G., Vitry, C.; Structure simulation of two ellagitannins from Quercus robur L. *Phytochemistry* **39,** 1193-1199, 1995.
15. Gutterres, M.; Analysis of vegetable tannin absorption during the tannage of hide/hide powder. *JALCA* **102**, 216-221, 2007.
16. Colak, S. M.; Soaking with tannins: The biocidal activity of vegetable tannins used in the soaking float. *J. Soc. Leather Tech. Chem.* **90**, 193-196, 2006.
17. Black, S. D., Mould, D. R.; Development of hydrophobicity parameters to analyze proteins which bear post- or cotranslational modifications. Anal. Biochem. **193**, 72-82, 1991.