Effects of Pretanning Processes on Bovine Hide Collagen Structure

by

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Abstract

The US meat industry currently produces approximately 35 million cattle hides annually as its most valuable coproduct. These hides serve as raw material, first for the leather industry, and then for the gelatin, and biomaterials industries. The conversion of animal hides into leather is a multistep process that has evolved more as art form than as science. Economic or environmental issues typically dictate changes in beamhouse processes that prepare the hide for tanning. The tanner evaluates these changes, in terms of impact on tannery costs and quality of leather produced. Thus far, the effects of beamhouse processes on the molecular characteristics of collagen have received little attention. The basis for tanning and most biomaterials applications is the stabilization of the collagen matrix, thus any changes to the molecular characteristics of hide collagen may be expected to impact these applications. This study showed that while the effects of different dehairing processes on the structure and stability of monomeric collagen were similar, the effects on the collagen fiber structure were distinct. These results 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 when beamhouse processes are altered.

Resumen

La industria de la carne de EE.UU. produce en la actualidad aproximadamente 35 millones de cueros vacunos al año como su coproducto de mayor valor. Estas pieles sirven como materia prima, en primer lugar para la industria del cuero, y luego para las industrias de gelatina y de biomateriales. La conversión de la piel animal en cuero es un proceso de múltiples pasos que ha evolucionado más como forma de arte que de ciencia. Cuestiones económicas o ambientales normalmente dictan los cambios en los procesos de ribera que preparan la piel para el curtido. El curtidor evalúa estos cambios, en términos de impacto sobre los costos y la calidad del cuero producido. Hasta ahora, los efectos de los procesos de ribera en las características moleculares de colágeno han recibido poca atención. La base para el curtido y para la mayoría de las aplicaciones de biomateriales es la estabilización de la matriz del colágeno, por lo que cualquier cambio en las características moleculares del colágeno puede impactar en estas aplicaciones. Este estudio mostró que si bien los efectos de diferentes procesos de depilación, en la estructura y en la estabilidad del colágeno monomérico fueron similares, los efectos sobre la estructura de la fibra de colágeno fueron distintos. Estos resultados se anticipan para asistir al curtidor, así como en la fabricación de biomateriales a base de colágeno y gelatina, para entender mejor su sustrato y los cambios que pueden ocurrir cuando los procesos de ribera son alterados.

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INTRODUCTION

The US meat industry currently produces approximately 35 million cattle hides annually as its most valuable coproduct. These hides serve as raw material, for the leather industry and ultimately, the gelatin, and biomaterials industries. Tanning, the conversion of animal hides into leather, is a multistep process that continues to evolve. Tanners adjust their processes in response to the availability of new formulations from their suppliers, or in anticipation of new regulations on currently used chemicals.[1] A seemingly minor change in one part of a beam-house process may lead to additional changes in later stages. The tanner accommodates to the changes and evaluates them in terms of their impact on tannery costs and quality of leather produced.

Thus far, the effects of beam-house processes on the molecular characteristics of collagen have received little attention. The basis for tanning and collagen-based biomaterials is the stabilization of the collagen matrix, thus any changes to the molecular characteristics of hide collagen may be expected to impact these applications. Gelatin and biomaterial manufacturers, who obtain their raw material from the tanner after some of the early hide preparation steps have been completed, are beginning to ask about the effects of changing processes on their raw material.

In a previous study,[2] we developed a protocol for the preparation of powdered hide to be used in research studies. In this study, we evaluate some of the effects of changing pretanning processes on the structure and stability of the powdered hide prepared by this protocol and on collagen extracted from powdered hide. As an additional reference point, we included a sample of commercial hide powder. The results are anticipated to assist the tanner as well as the manufacturers of collagen-based biomaterials and gelatin to better understand their substrate and changes in it that may occur when beam-house processes are altered.

EXPERIMENTAL

Materials

Fresh hide was obtained from a local abattoir, cut into pieces (~15 × 40 cm) and used to prepare powdered hide. Commercial hide powder, insoluble collagen, pepsin (porcine gastric mucosa), and bacterial collagenase (361 units/mg) isolated from *Clostridium histolyticum*, were obtained from Sigma-Aldrich, St. Louis, MO. Rat tail, type 1 collagen, was obtained from Millipore, Billerica, MA. All other chemicals were reagent grade from various suppliers.

Sample preparation

Powdered hide

The cut pieces of hide were divided into two sets. One set was sulfide dehaired and relimed essentially as described by Cabeza et al.[3] The other set was dehaired by an oxidative process as described by Marmer and Dudley[4] and then relimed as described by Cabeza et al.[3] The relimed hide pieces were split to a thickness of 2.0 mm and washed in a drum with running water for 1 h. They were then tumbled for 3 h in a 400% float adjusted to pH 7.1 by the addition of 0.5% acetic acid (unless noted otherwise, all added chemicals are based on the weight of the limed split). The pH was adjusted to 5.4 by the addition of 0.5% acetic acid to the drum and the split tumbled for 4 h, then washed in running water for 30 min and refrigerated overnight. On the following day, the splits were tumbled twice for 2 h in 400% water, containing 4.35% NaCl pH 6.6, and then washed for 1 h with water. They were treated 3 times with 0.3% lime in 400% float for 2 h at pH 11.5-11.7. The splits were then washed for 1 h with water, followed by tumbling overnight in 400% float containing 2.3% acetic acid and 3.2% sodium acetate. The next day, they were tumbled at least 3 times in 400% water for 1 h until the pH of the float was the same as that of the water. Each split was then cut into strips and stored frozen until they could be acetone dried. Thawed strips were dried with acetone, in a fume hood, in small batches. The pieces were weighed and mixed with 400% acetone, and allowed to sit overnight. This step was repeated, 5 - 7 times, until the specific gravity of the float was the same as that of acetone, and no further water could be removed. The hide pieces were then air-dried, ground in a Wiley mill and stored under refrigeration in resealable plastic bags.

Extracted collagen

Collagen was extracted from powdered hide samples by adding 250-500 mg of sample to 100 ml of 0.5 M acetic acid and stirring for 3 days at 4°C. The samples were centrifuged and the supernatant lyophilized. The lyophilized product was dissolved (3 mg/ml) in 0.05 M acetic acid and dialyzed overnight against two changes of 0.05 M acetic acid. Concentrations of extracted collagen were estimated from the absorbance at 218 nm using the absorption coefficient 9.43 cm^{-1}-ml-mg^{-1} determined by Na.[5] The samples were centrifuged and stored at 4°C for later analysis.

Analyses

Moisture content of powdered hide was determined from the weight loss when a sample was heated in a vacuum oven at 50°C for 16 h and then cooled in a desicator.[6] Dried samples were further heated in a muffle furnace at 600°C for 2 h to determine ash content.[6] Nitrogen content was determined in triplicate by the Kjeldahl method according to AOAC 920.39.[7] A system comprising a Tecator digestion unit and a Kjeltec 8100 distillation unit (Foss North America, Eden Prairie, MN) was used for the analysis, and titration was done manually. A

factor of 5.13 for the conversion of nitrogen to collagen was calculated as described previously.[2]

Scanning Electron Microscopy (SEM)

Powdered hide samples were mounted on the surface of carbon adhesive tabs with the help of an all purpose glue (Devcon® Duco Cement). Silver paint was applied to the exposed surface area around the sample to ensure conductivity and the entire specimen was sputter-coated with gold using a Scancoat Six Sputter Coater (Edwards, Wilmington, MA). Samples for SEM were imaged using a Quanta 200 FEG environmental scanning electron microscope, (FEI Company, Hillsboro, OR). SEM imagines were captured as described by Brown, et al.[8]

Susceptibility to collagenase

Susceptibility to cleavage by collagenase was determined by a modification of the Mandl method.[8,9] Samples from each treatment were analyzed in triplicate. Dry powdered hide samples were suspended in 0.05 M tris(hydroxymethyl)-methyl-2-aminoethane sulfonate (TES) buffer with 0.36 mM calcium chloride, pH 7.5, and incubated with collagenase at a ratio of 1 mg collagenase to 100 mg powdered hide, for 5 h at 37°C. The resulting solution was reacted with ninhydrin-citric acid and the absorbance read at 600 nm on a UV-Vis spectrophotometer. Susceptibility to collagenase is reported in terms of μmoles of amino acid released per mg of hide powder (dry weight) as compared with a standard curve for leucine.

Electrophoresis

Powdered hide samples were digested with pepsin (1 mg pepsin + 100 mg powdered hide)/ml overnight at 4°C.[10] The digested samples were centrifuged at room temperature for 0.5 h at 1000 RPM, and the supernatant, was dialyzed, in Slide-A-Lyzer dialysis cassettes (10,000 MWCO) 3-12 ml capacity (Thermo Scientific, Rockford, IL) for two days against four changes of 0.5 M acetic acid, and then lyophilized. Solubilized powdered hide samples were prepared for SDS-PAGE (polyacrylamide gel electrophoresis in sodium dodecyl sulfate) as described by Taylor et al.[11] Separation was achieved on a PhastGel System (GE Life Sciences, Piscataway, NJ) using an extended protocol to assure that intact collagen could move into the gel. A broad range SDS-Standard (Bio-Rad, Hercules, CA) containing nine proteins ranging in size from 6,500 to 200,000 Daltons, and type I soluble and insoluble collagens (Sigma, St. Louis, MO) were included as controls. A second larger format gel was run, and isolated protein bands tested qualitatively for hydroxyproline using a Hydroxyproline Assay Kit (BioVision, Mountain View, CA).

Hydrothermal stability of powdered hide

Hydrothermal stability of powdered hide was determined on a Multi-Cell Differential Scanning Calorimeter (DSC) (model CSC-4100) from Calorimetry Sciences Corporation, Lindon, UT, as previously described.[12] Powdered hide samples were prepared for DSC experiments by soaking in distilled water overnight and then blotting on filter paper. Moist, blotted samples (100 - 250 mg) were weighed into ampoules that were sealed and placed in the calorimeter. The calorimeter was programmed to record heat flow as μcal/°C while the temperature was increased from 30°C to 130°C at 1.5°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. Samples were dried, and the initial moisture content (usually ~200%) was calculated.

Thermal stability of extracted collagen

The thermal stability of soluble collagen was determined from the circular dichroism spectrum as described previously.[13] Samples, 300 μl in volume, containing ~1 mg/ml extracted collagen in 50 mM acetic acid were placed in 1-mm-pathlength quartz cuvettes that were fitted with Teflon stoppers to prevent evaporation during the melting experiments. Filled cuvettes were placed in the refrigerator for at least 12 hours to maximize the formation of the triple-helix structure in the sample. The ultraviolet (UV) spectrum of the sample was scanned from 300 to 190 nm against a reference solution containing all components except collagen. The thermal stability of the triple helix was determined by following the change in the circular dichroism (CD) signal at 223 nm (AVIV 420 Spectropolarimeter, AVIV Biomedical Inc., Lakewood, NJ) with increasing temperature. An initial scan of the sample was made at 10°C between 250 and 200 nm. The CD was then programmed with a scan-melt-scan algorithm. Melting curves were obtained by recording the CD signal at 223 nm every 0.5°C between 10°C and 60°C at a rate of ~6 min per degree. The CD signal was read in mdeg and converted to molar ellipticity as shown below:

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

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 (T_p and T_d) were obtained from the derivative of the melting curve.

RESULTS AND DISCUSSION

Moisture, ash, protein content, and hydrothermal stability of freshly prepared powdered hide from sulfide and oxidative dehaired hide are summarized and compared with those for a commercial hide powder in Table I. Triplicate results on a single sample are in reasonable agreement for a natural material. Moisture values (9% - 14%) are in line with our previous powdered hide preparations.[2] Ash content on a moisture free basis was uniform (0.1 - 0.2%), and lower than the 0.5 - 1% reported for some commercial hide powders.[14]

TABLE I
Analysis of Powdered Hide

Sample[a]	Moisture, %	Ash[b], %	Protein[b], %	Ts[c], °C
Oxidative	9.4±0.4	0.10±0.01	92.07±0.39	66.4 ±0.42
Sulfide	13.9±0.1	0.21±0.11	84.52± 0.59	64.0 ±0.56
Hide Powder	9.7±0.5	0.12±0.09	84.52± 0.65	66.9 ±0.21

[a]Each sample was analyzed in triplicate.
[b]On a dry weight basis
[c]On a wet sample basis, moisture content approximately 200% of dry sample weight.

Protein content, calculated from total nitrogen as type I collagen, is significantly higher (92% to 84%) in the powdered hide from oxidative dehairing, than in powdered hide from sulfide dehairing, or the commercial hide powder.

Scanning electron microscopy (SEM)

SEM images of powdered hide captured at 1000x magnification (Figure 1) show fiber bundles with individual fibers separating at the edges. The SEM image at 1000x of our oxidative dehaired powdered hide (Figure 1a), shows large fiber bundles (2 – 5 μm) with relatively open structures, where individual fibers with diameters in the range of 0.5 – 0.8 μm can easily be discerned. In contrast, 1000x SEM images of our sulfide dehaired powdered hide (Figure 1b) and the commercial hide powder (Figure 1c) show fiber bundles that are more compact with fewer distinguishable fibers.

Susceptibility to collagenase

The fibrillar nature of collagen provides considerable resistance to attack of the triple helical structure by most proteases. 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 buried sidechain groups as well as by creating additional N-terminal amino groups. Under the digestion conditions used in this study, the number of available primary amino groups was essentially doubled by the collagenase treatment on soluble collagen and our powdered hides (Figure 2). The number of available amino groups, after collagenase digestion, expressed as (μm/mg dry powdered hide) is about 50% of the number for soluble collagen, suggesting that the fibrous nature of the hide provides some protection against collagenase. Interestingly, although the percent increase in available amino groups after collagenase treatment of the commercial hide powder was less than for the other samples, the final value was the same as for our sulfide dehaired material, suggesting that the difference is in the availability of amino groups prior to digestion, a value that was considerably more variable in the commercial hide powder than in our powdered hide samples.

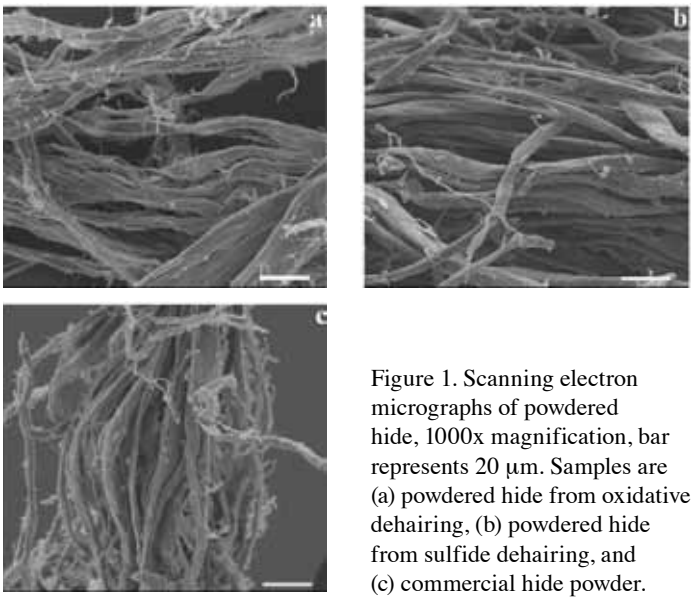

Figure 1. Scanning electron micrographs of powdered hide, 1000x magnification, bar represents 20 μm. Samples are (a) powdered hide from oxidative dehairing, (b) powdered hide from sulfide dehairing, and (c) commercial hide powder.

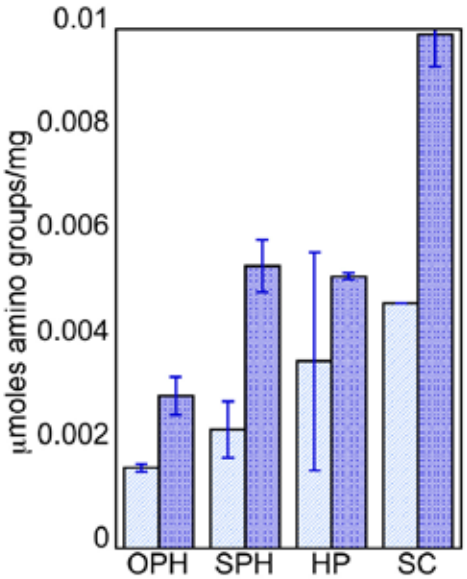

Figure 2. Available primary amino groups before (light blue) and after (darker blue) collagenase treatment of (OPH) powdered hide from oxidative dehairing, (SPH) powdered hide from sulfide dehairing, and (HP) commercial hide powder, and soluble collagen (SC).

Electrophoresis

Pepsin treatment of collagen cleaves the native crosslinks in the telopeptide region, not the triple helix, to solubilize collagen. Samples of pepsin treated powdered hide from sulfide dehairing, commercial hide powder, and insoluble collagen gave essentially identical SDS-PAGE patterns, Figure 3. The patterns are similar to those reported by Lin and Liu,[15] with major bands for the collagen β-chain near 200 kDa, and the α1- and α2- chains slightly above the 116 kDa marker. Lower molecular weight bands seen in the 35 - 45 kDa region of the powdered hide from oxidative dehaired hide, lane 5, tested negative for hydroxyproline and appear to be smaller protein components of the extracellular matrix, not collagen fragments. These smaller proteins that are not present in powdered hide from sulfide dehairing, or in the commercial hide powder, contribute to the overall higher protein content of the powdered hide from oxidative dehairing, as well as to the lower number of available amino groups after collagenase treatment. A 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.

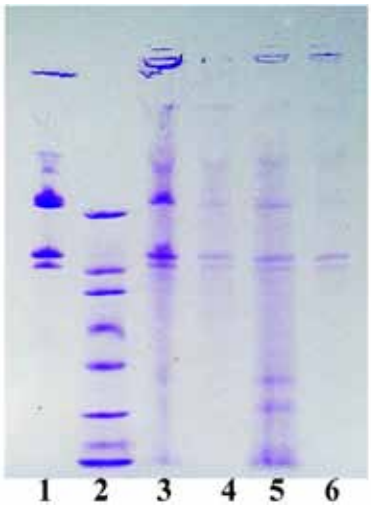

Figure 3. SDS-PAGE gel, 4 - 15% gradient. Lane (1) soluble collagen, (2) a broad range molecular weight standard, (3) commercial hide powder, (4) sulfide dehaired powdered hide, (5) oxidative dehaired powdered hide and (6) a commercial insoluble collagen.

Hydrothermal stability of powdered hide

The apparent shrinkage temperature determined by DSC of 64.0±0.56°C for our sulfide dehaired powdered hide was in agreement with the 64.8±1.2°C determined in our earlier study.[2] Apparent shrinkage temperatures for powdered hide from oxidative dehairing and the commercial hide powder were slightly above 66°C (Table I), comparable to the shrinkage temperatures for raw-hide measured by Komanowsky[16] in a shrink-temperature apparatus as described by Fein et al.[17] Factors that contribute to differences between the shrinkage temperatures determined for a strip of hide and for powdered hide include the exposed surface area, the degree of hydration and the rate of heating. DSC is the most widely used research tool for monitoring thermal stability of collagen in powdered hide. In the DSC configuration used for this study, the powdered hide is well hydrated, and the heating rate of 1.5°C per min in these experiments is comparable to the heating rate for hide strips.[16] Our results suggest that pilot scale sulfide dehairing may be more damaging to hide structure than pilot scale oxidative dehairing or sulfide dehairing on an industrial scale.

Thermal stability of extracted collagen

The CD spectrum at 10°C of a typical soluble collagen at pH 4.3 in 50 mM HAc[13] consists of a positive band around 223 nm and a stronger negative band at 198 nm characteristic of the collagen triple helix.[18] These features are best observed with solutions that have been cooled to below 15°C for several hours. At higher temperatures, the helical conformation is lost and the CD spectrum becomes featureless. CD spectra (not shown) of the collagen extracted from partially processed powdered hide were similar in shape to those for soluble collagen, but with less intense positive and negative bands. The melting curves recorded for the helix to unordered transitions of soluble collagens[13] consisted of a flat region $[\theta]_{223nm}$ ~ 5500 deg cm^2dmol^{-1} at temperatures below 20°C representing completely helical protein, and a second flat region $[\theta]_{223nm}$ ~ -1400 deg cm^2dmol^{-1} at temperatures above 40 °C representing completely unfolded protein. Between these two regions, the temperatures associated with the pretransition (T_p) and denaturation (T_d) were most clearly seen in the derivatives of the melting curves. Although melting curves (Figure 4) for collagen extracted from our powdered hide samples and the commercial hide powder were qualitatively similar to those reported for soluble collagen, the maximum helical structure achieved when our collagen from oxidative dehairing or the commercial hide powder was cooled to 10 °C was about 70% of that obtained with soluble collagen. In addition to values for T_p and T_d (Table II) that are in good agreement with those for soluble collagen,[13] the derivative curves for these samples show an inflection point near 50 °C, the significance of which has not yet been established. Conversely, although extracted collagen from our sulfide dehaired powdered hide was initially even less helical, at 35% of full helix, the derivative curve is indicative of a classical two-stage helix to unordered transition.

In the present study, a comparison of powdered hide from the oxidative dehairing method of Marmer and Dudley[4] with powdered hide from the sulfide dehairing described by Cabeza et al.[3] and with a commercial hide powder showed little difference in the collagen monomer (triple helix) as evidenced in the melting profiles. At the fiber level, our sulfide dehaired material appeared strikingly similar to the commercial hide powder. Our oxidative dehaired material had a more open fiber structure, suggesting greater ease of penetration for chemicals as well as perhaps more available binding sites.

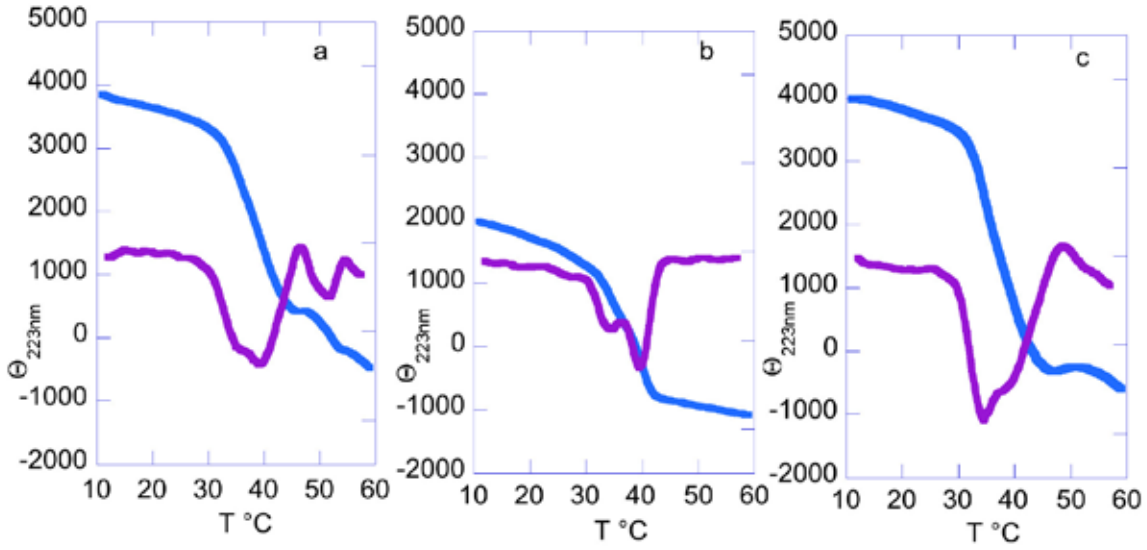

Figure 4. Melting curves (blue) and derivative curves (magenta) for extracted collagen from (a) oxidative dehaired hide, (b) sulfide dehaired, and (c) commercial hide powder. Molar ellipticity at 223 nm recorded every 0.5°C over the 10-60°C range with a time constant of 5 s and a 10°C/h heating rate.

Based on total nitrogen, the material from oxidative dehairing contained more protein than the sulfide dehaired material, however, the SDS PAGE results suggest that this is due to incomplete removal of noncollagenous proteins.

CONCLUSIONS

Tanning processes continually evolve in response to environmental, economic and legislative pressures. Changes, in a process in response to a request to eliminate or minimize the use of one or more of the traditional chemicals, may have unanticipated effects further into the tanning process and on the characteristics of the final leather produced. An understanding of the mechanisms of tanning depends on knowledge of the effects on the hide substance of beamhouse processes that precede the tanning step. Likewise, manufacturing processes for collagen-based biomaterials and technical gelatins must be based on the results of changes to the starting material caused by new pretreatments of the hides. The results of this study show some distinct differences in the condition of the collagen fibers in powdered hide from two different dehairing processes. Because multiple variations of processes such as dehairing are in practice and evolving throughout the industry, an awareness of the possible effects on collagen fiber structure could be valuable.

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TABLE II
CD and Thermal Transition Parameters for Extracted Collagen

Sample[a]	mg/ml[a]	Helix[b]	Unordered[b]	Tp[c], °C	Td, °C
OX (3)	0.2-0.9	2200 ± 1400	-800 ± 300	33.5 ± 1.9	38.2 ± 5.5
SU (3)	0.3-0.9	2100 ± 400	-1200 ± 200	33.6 ± 0.9	39.5 ± 0.6
CHP (3)	0.8-1.0	3500 ± 1000	-900 ± 200	33.6 ± 1.0	37.6 ± 0.7
SC (16)	0.8-2.1	5600 ± 400	-1400 ± 150	32.5 ± 2.4	38.7 ± 2.2

[a]Samples of extracted collagen from powdered hide (OX) oxidative dehaired, (Su) sulfide dehaired, (CHP) commercial hide powder are compared with (SC) soluble collagen[13]. Numbers in parentheses are the number of CD experiments per sample. Concentrations were determined spectrophotometrically.
[b]Molar ellipticities at 223 nm for helix were determined at 10°C, unordered structure at 60°C.
[c]T_p and T_d are the pretransition and denaturation temperatures, respectively.

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